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DEVELOPMENT OF A LABORATORY BASED SYSTEM FOR SELECTING
INSECT PATHOGENIC FUNGI WITH GREATEST POTENTIAL FOR SUCCESS IN
THE FIELD

by

Chad Alton Keyser

A dissertation submitted in partial fulfillment
of the requirement for the degree

of

MASTER OF SCIENCE

in

Biology

Approved:

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Logan, UTAH

2010

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ABSTRACT

Development of a Laboratory Based System for Selecting Insect Pathogenic Fungi with
Greatest Potential for Success in the Field

by

Chad Alton Keyser, Master of Science

Utah State University, 2010

Academic Professor: Dr. Edward W. Evans
Research Professor: Dr. Donald W. Roberts
Department: Biology

Many insects are important agricultural pests, and active control is necessary to keep them at abeyance. The naturally occurring entomopathogenic fungus *Metarhizium* is a promising tool to control pest insects, and its use avoids the well-known harmful side effects of chemical pesticides. Thousands of unique isolates of *Metarhizium* exist throughout the world. These isolates vary widely in their ability to cause infection and to tolerate stressful habitats. The research reported here tests the THESIS: A laboratory-based system can be devised that identifies, from among many *Metarhizium* isolates, those isolates with the greatest potential for successful biological control of pest insects in the field. The study was built on the testing of four hypotheses: (1) Laboratory bioassays using target pest insects will distinguish highly virulent strains of *Metarhizium* from less virulent strains, (2) Quantity and quality of mass-produced pathogenic fungi will vary among species and strains of *Metarhizium*, (3) The tolerance to ultraviolet

radiation will vary among species and strains of *Metarhizium*, (4) The effect of temperature on growth rates and survival of both *Metarhizium* spores and hyphae will vary among isolates and species. These hypotheses test four field-relevant traits using a panel of ten isolates of *Metarhizium* isolates.

Seven sets of laboratory experiments were devised to define the range of responses within the traits covered by the hypotheses. This series of general laboratory tests was developed to assist in identifying fungal isolates with high potential for field use. These tests included evaluation of each isolate's (a) insect pathogenicity, (b) mass-production capabilities, (c) tolerance to high temperatures, (d) tolerance to UV-B radiation, (e) rate of vegetative growth, (f) rate of spore germination, and (g) an evaluation of presence or absence of a post-stress growth inhibition. The application of this protocol to the isolates used in this study indicates that four isolates have high field potential, i.e., DWR 203, DWR 346, DWR 356 and ARSEF 324, and three of these were tested in a field trial. By following the procedures outlined in this thesis, selection of "good" isolates can be accomplished in the laboratory, and a successful isolate can be identified from the abundance of isolates present in nature.

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A hundred times every day I remind myself that my inner and outer life depend on the labors of other men, living and dead, and that I must exert myself in order to give in the same measure as I have received and am still receiving.

Albert Einstein

Through this acknowledgment, I express my sincere gratitude to all those who have supported, encouraged, mentored, and helped as I have traversed the path to completing this thesis; truly these individuals made the experience worthwhile.

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Chad Alton Keyser

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Insects are of paramount human consequence, “our history, health and finances all pivot to a surprising degree on [them]” (Marshall, 2006). It is estimated that insects outnumber humans 200 million to one, and there are 40 million insects for each acre of land. In the Brazilian Amazon alone it is estimated that ants outweigh the total biomass of vertebrates by four to one. Based on either numbers or biomass, insects are the most successful animals on earth (Pedigo, 2002). While the sleepless might dream of nights free from insect noise, scenarios involving the absence of insects would alter nearly all levels of life, for example, loss of consumer products such as honey and silk, the disruption of food production systems from the absence of pollinators, the collapse of insect-dependent food chains, and even as far-reaching as the upset of insect-enhanced microbial decomposition. While human dependence on insect existence is undeniable, often insects are seen as an archaic adversary intent on vexing and plaguing mankind. More than just creepy six-legged home-invaders, insects can be vectors of severe human diseases, such as malaria and the plague (Blanford et al., 2005). Furthermore, a territorial battle has raged between man and insect since the early cultivation of crops and domestication of livestock. Without question, active insect-pest control is necessary. While the sophistication of our weapons for controlling insects has increased with time, insects are favored by rapid evolution and overwhelming numbers.

As our understanding of the complexity and fragility of ecosystems has increased, we have come to recognize the delicate balance between controlling pest insects and maintaining beneficial insects. To this end, a stratagem known as Integrated Pest

Management (IPM) was developed 50 years ago, and its use currently is widely recommended by pest control experts. IPM systems focus on reducing losses in an ecologically, as well as economically, sound manner. They encourage management programs that integrate a wide array of control methods, including pesticides, host-plant resistance, tillage, sanitation and biological control (Kogan, 1998; Pedigo, 2002).

The Mormon cricket (MC) (*Anabrus simplex*), is a gregarious perennial orthopteran pest common to the Western United States that has been known to cause serious agricultural problems. The MC (actually a katydid, closely related to grasshoppers) is a large, flightless insect with jumping legs and the ability to migrate many miles during its lifetime of up to six months. It can weigh up to six grams (0.2 oz.) and measure as much as 60 mm (2 in.) in length (MacVean, 1990; Pfadt, 1994; Gwynne, 2001; Bailey et al., 2005; Lorch et al., 2005; Simpson et al., 2006). Generally, MC occur in low numbers and cause relatively insignificant damage to crops; but, unfortunately, they sometimes reach outbreak population levels that can last up to 15 years (Pfadt, 1994). During these outbreaks, the high MC numbers may lead to economically significant losses to farmers and ranchers.

Currently, the primary means for controlling MC and other orthopteran pest insects are broad-spectrum chemical pesticides. In the United States, carbaryl, malathion and dimilin in wheat-bran baits and sprays are the most commonly employed chemicals. These chemicals are effective at suppressing pest populations, but they have many disadvantages. These include that they are prohibited in many areas, such as near waterways and habitats of threatened or endangered species, their use by organic farmers

and ranchers is not allowed, they are under continual review by government and private agencies for any unforeseen harmful side-effects to humans or other mammals, and they generally have a short effective period after field application. Furthermore, broad-spectrum insecticides often will affect non-target beneficial insects, including pollinators, resource producers (honey, silk or wax), natural enemies of insect pests, wildlife food, and scavengers (Murphy et al., 1994; Peveling et al., 1999; Lomer et al., 2001). These deleterious effects on beneficial insects are of major concern, and in some cases, may cause greater ecological and economical damage than an uncontrolled pest.

As previously mentioned, controlling pest-insect populations is often a tricky and complex problem; the tactics employed by IPM programs involve balancing benefits and drawbacks associated with certain treatments. Orthoptera-control programs in the United States would benefit by the addition of alternative control methods (Lomer et al., 2001). One alternative to chemicals that has shown well-founded potential for use against orthopteran pests is biological control (Thomas, 1999). Biological control, defined as “the use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be” (Eilenberg et al., 2001), includes the use of pest predators, parasitoids, parasitic nematodes, bacterial pathogens, viral pathogens, fungi, and microsporidia (Goettel and Johnson, 1997; Hajek, 2004; Vincent et al., 2007).

The entomopathogenic fungi *Metarhizium* spp. are common insect pathogens, and they are some of the most promising fungi for biological control (Thomas et al., 1995; Enserink, 2004; Roberts and St. Leger, 2004; Alston et al., 2005; Blanford et al., 2005).

Metarhizium spp. have been found in soil or on dead insects throughout the world. In fact, they have been isolated from every continent except Antarctica (Roddam and Rath, 1997; Roberts and St. Leger, 2004). Most *Metarhizium* species, including *M. anisopliae*, *M. robertsii*, and *M. brunneum*, are pathogenic towards a wide range of invertebrates. However, at least one species, *M. acridum*, is host specific, in that it targets only some species of orthopteran insects (Driver et al., 2000; Bischoff et al., 2009). Due to the current level of interest in *Metarhizium* research and the advancement of scientific technology, the taxonomy of *Metarhizium* spp. is in the process of being resolved. The systematization of several new species within the genus *Metarhizium* was recently reported (Bischoff et al., 2009). Isolate identification is based primarily on molecular DNA sequencing. The placement of most isolates therefore is not expected in the near future. Accordingly, isolate nomenclature in this study not identified as prescribed by Bischoff et al. will be referred to as *M. anisopliae sensu lato* (s.l.).

In the United States only two isolates of *Metarhizium* have been registered commercially and are not widely used as biocontrol agents. At many locations outside the U.S., including Africa, Australia, China and Europe, insect control programs using *Metarhizium* have been implemented with favorable results. Many of these programs utilize the host specific variety, *M. acridum*, to control orthopteran pests (Roberts and St. Leger, 2004; Faria and Wraight, 2007).

One disadvantage to the use of fungal biological control agents is that they are susceptible to environmental factors such as temperature and solar radiation. Thousands of isolates of *Metarhizium* have been isolated and placed in culture collections, and these

isolates vary greatly in tolerances to environmental factors, host specificity and virulence towards insects (Humber and Hansen, 2009) . Historically, fungal isolates used for biocontrol were selected for commercialization, primarily based on two traits: their virulence to a target insect and ease of mass production (Schaeffenberg, 1964; Bateman et al., 1996; Roberts and St. Leger, 2004). While both of these traits are important in the selection process, there are examples where fungal isolates with high virulence in laboratory tests were quite ineffective in the field (Butt et al., 2001; Roberts and St. Leger, 2004). Accordingly, expanding the selection criterion will be necessary to identify isolates that have the greatest potential for use as insect-pest control agents.

Thesis: A laboratory-based system can be devised that identifies, from among many *Metarhizium* isolates, those isolates with the greatest potential for successful biological control of pest-insects in the field. To accomplish this aim the following four hypotheses will be tested:

Hypothesis 1: Laboratory bioassays of target pest insects will distinguish highly virulent strains of *Metarhizium* from less virulent strains.

Background

Host mortality rates and levels differ between strains and species of *Metarhizium* and various insect hosts. *In-vitro* assays are a useful tool in evaluating the virulence of each isolate and selecting the most promising for further experimentation. Isolate pathogenicity and virulence tests are often one of the primary focal points of candidate selection for commercial development.

While few studies have been conducted involving entomopathogens and *A. simplex*, MC have been shown to be susceptible to *M. brunneum*, *M. robertsii* and *M. acridum* as well as several isolates of *M. anisopliae* s.l. (Roberts et al., 2007). In addition, other orthopteran pests, primarily grasshoppers and locusts, have been the focus of many virulence assays utilizing *Metarhizium* (Milner and Prior, 1994; Prior et al., 1995; Bateman et al., 1996; Caudwell and Gatehouse, 1996; Milner et al., 1996, 2003; Delgado et al., 1997; Fargues et al., 1997; Milner, 1997; Peveling and Demba, 1997; Thomas and Jenkins, 1997; Lomer et al., 2001; Fagade et al., 2005; Entz et al., 2008). Several of these studies have included ARSEF 324 (also incorporated in this dissertation), an isolate found to be among the most virulent *M. acridum* isolates toward locusts and grasshoppers (Milner and Prior, 1994; Thomas et al., 1995; Milner et al., 2003)

Since virulence studies assess the ultimate goal of biological control (mortality), they are useful in evaluating the effect of many different treatments. For example, using virulence assays, studies have appraised the following: the effect of the nutrient source used in conidial production (Ibrahim et al., 2002; Shah et al., 2005; Rangel, 2006; Rangel et al., 2008a), the effects of temperature on virulence (Fargues et al., 1997; Thomas and Jenkins, 1997; Brooks et al., 2004), the effect of moisture content prior to conidial storage (Moore et al., 1996), the effect of pathogen dose (Milner and Prior, 1994; Thomas and Jenkins, 1997; Milner et al., 2003; Fagade et al., 2005), the effect of whether repeated sub-culturing affects virulence, and if so whether it can be restored through insect passage (Daoust and Roberts, 1982), the effect of insect thermal regulation,

including behavioral fever, on virulence (Blanford and Thomas, 2001; Ouedraogo et al., 2002; Ouedraogo et al., 2004), effects of fungal formulation (Prior et al., 1995; Thomas et al., 1995; Caudwell and Gatehouse, 1996), and pathogenicity of isolates towards non-target host insects (Peveling and Demba, 1997).

While laboratory virulence tests are not necessarily well correlated with field effectiveness (Roberts and St. Leger, 2004), isolate selection for commercialization routinely emphasizes laboratory virulence (Bateman et al., 1996; Bugeme et al., 2008).

Hypothesis 2: Quantity and quality of mass-produced pathogenic fungi will vary among strains and species of *Metarhizium*.

Background

Some chemical pesticides are relatively cheap and stable, with long shelf-lives. To effectively compete with chemicals, fungi must demonstrate an ability to produce large amounts of viable conidia on an inexpensive substrate, and afford long-term storage (long shelf life). While liquid fermentation is more advanced than solid substrate fermentation and would be more cost efficient for industry, *Metarhizium* conidiospores are more environmentally persistent when mass produced by solid substrate fermentation in comparison to liquid fermentation (Jenkins and Goettel, 1997; Lomer et al., 2001; Roberts and St. Leger, 2004). The nutrient sources must provide adequately balanced levels of carbon, phosphorous and nitrogen. Solid-growth substrates commonly used are agricultural grains, seeds or beans such as rice bran, wheat bran, and barley flake (Cherry et al., 1999; Lomer et al., 2001; Roberts and St. Leger, 2004). Apart from a nutrient source, growth requirements also include adequate temperature, pH, water activity, and

gas exchange of CO₂ and O₂ (Roberts and St. Leger, 2004; Grace, 2005). Another challenge is maintaining a sterile work environment, which is crucial since *Metarhizium* is often out-competed by other fungi and bacteria. To accommodate these requirements, mass production is usually accomplished in autoclavable polypropylene bags with a filtered gas exchange system (Cherry et al., 1999; Roberts and St. Leger, 2004; Grace, 2005).

After fermentative growth is complete, the substrate plus fungus is harvested. This step may be accomplished in several ways. For example, conidia and infested grain can be milled and the resulting powder be applied to the field usually as an aqueous suspension, for pest biocontrol. Alternatively, conidia can be removed from the grain. Separating the spores from the growth substrate can be done in several ways: floating in a water bath (conidia float while rice grains tend to sink), using metal sieves to separate conidia from grain, or using centrifugal commercial vacuum device, (Microharvester™) (Roberts and St. Leger, 2004; Grace, 2005). There are many published approaches to mass producing fungal conidia. It is likely that if one particular substrate or growth method does not produce sufficient spores of a particular isolate, adjusting the production method may enhance quantity and quality. The important aspects to remember are that mass production needs to produce viable spores to be effective in the field, that an economically reasonable number of spores needs to be harvested and the conidia must be of high quality as to virulence, storability, and field efficacy.

Hypothesis 3: The tolerance to ultraviolet radiation will vary among species and strains of *Metarhizium*.

Background

An important obstacle to field use of fungal pathogens against insect-pest control is their susceptibility to ultraviolet (UV) radiation which has been shown to reduce viability after only a short period (usually a few hours) of direct exposure to sunlight (Alves et al., 1998; Braga et al., 2001a, 2001b, 2001c, 2002, 2006; Miller et al., 2004; Rangel et al., 2004, 2005b; Ghajar et al., 2006; Fernandes et al., 2007). The fraction of the ultraviolet spectrum that is not filtered out by earth's atmosphere is composed of UV-A (wavelength 320-400nm) and UV-B (280-320nm). Of the two fractions, UV-B is considered more biologically damaging because of its direct interaction with DNA. UV-A, which can still cause damage, does so through oxidation (Griffiths et al., 1998; Braga et al., 2001b; Roberts and St. Leger, 2004). *Metarhizium* is susceptible to both UV-A and UV-B which can cause delayed germination after only 1 hour of exposure and significant reduction in viability after only 2 hours (Zimmermann, 1982; Braga et al., 2001a; Braga et al., 2001c; Rangel et al., 2004; Rangel et al., 2006a; Rangel, 2006). Some isolates of *M. acridum*, like ARSEF 324, are more tolerant to UV radiation and have greater than 50% germination after 4 hours of exposure (Braga et al., 2001c; Rangel, 2006).

Selecting or developing formulations for isolates with increased tolerance to UV radiation could improve field effectiveness. Braga et al. (2006) noted that wild type green conidia were more tolerant to UV radiation than mutants that had changed or lost their color pigmentation. Rangel et al. (2004; 2006a) demonstrated that UV tolerance

was increased twofold after producing *Metarhizium* conidia spores on nutritive-deprived media, and that conidia obtained from an insect cadaver was more susceptible to UV than *Metarhizium* conidia produced on nutrient-rich artificial media.

Hypothesis 4: The effect of temperature on growth rates and survival of both *Metarhizium* spores and hyphae will vary among isolates and species.

Background

The influence of temperature varies considerably according to the life requirements of an insect-killing fungus. Most *Metarhizium* species, generally considered to be mesophilic fungi, have severely restricted growth below 10°C and above 37°C (Schaeffenberg, 1964; Fargues et al., 1997; Ouedraogo et al., 1997; Thomas and Jenkins, 1997; Hallsworth and Magan, 1999; Rangel, 2006; Fernandes et al., 2010). In determining an isolate's preferred temperature, studies often focus on either conidial germination (Luz and Fargues, 1997; Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Yeo et al., 2003; Bugeme et al., 2008; Leemon and Jonsson, 2008), or vegetative (hyphal) growth (Ouedraogo et al., 1997; Ekesi et al., 1999; Hallsworth and Magan, 1999; Smits et al., 2003; Brooks et al., 2004). Optimal temperature for germination and vegetative growth for most *Metarhizium* isolates range between 25° and 30°C, and are often closely related (Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Yeo et al., 2003; Bugeme et al., 2008). Roberts and St. Leger (2004) noted that for a pathogen to infect a host it must be “in tune” with the target host's habitat. The MC resides in a climate typified by day temperatures up to 40°C and nights near freezing. Strain selection based on thermal tolerance may be critical in choosing an isolate for

biological control of this insect. Optimal infection temperature is not necessarily the same as optimal temperature for growth (Fargues et al., 1997; Thomas and Jenkins, 1997).

In addition to coping with fluctuating temperatures, insect pathogens must also overcome host behavioral defenses. For instance, an infected host may elicit behavioral fever in which it elevates its body temperature by basking in the sun in order to restrict or kill an infecting fungal pathogen (Parker, 1982; Kemp, 1986; Whitman, 1987, 1988; Prange and Pinshow, 1994; Thomas and Jenkins, 1997; Arthurs and Thomas, 2001; Blanford and Thomas, 2001; Ouedraogo et al., 2002, 2004; O'Neill and Rolston, 2007). Turnbow (1998) showed that MC do in fact exhibit a slight, yet statistically significant, behavioral fever ($\sim 0.5^{\circ}\text{C}$) when infected with *Beauveria bassiana*. Turnbow also noted that the preferred temperature of MC, near or above 35°C , may be sufficient to block fungal infection. Locusts and grasshoppers have been shown to have a preferred body temperature of 38° to 40°C , and when infected with a pathogen they may increase preferred temperature to 42°C (Carruthers et al., 1992; Blanford et al., 1998; Blanford and Thomas, 2000; Lomer et al., 2001; Ouedraogo et al., 2003). Realizing that an isolate's ability to survive high temperature probably significantly influences success of that isolate in field application, biological control, laboratory studies have been conducted to evaluate spore resistance to high temperatures, and calculate thermal death points of spores (the lowest temperature where all spores will be killed within 10 min). For *M. anisopliae* s.l. isolates, the thermal death point of conidia ranges between 45° and 60°C depending on the isolate (Zimmermann, 1982; Rangel et al., 2005b). In addition, *M.*

acridum isolate ARSEF 324 has been shown to tolerate (i.e., have more than 50% germination) 45°C wet heat for more than 12 hours, while *M. robertsii* isolate ARSEF 23 tolerated 45°C for only 4 hours (Rangel et al., 2005b; Fernandes et al., 2010).

Conclusion

Careful evaluation and comparison of *Metarhizium* isolates in the laboratory can greatly increase the potential for successful biological control, while simultaneously reducing time and cost of laborious field trials. Isolate screening programs, in addition to traditional virulence and culturability tests, should include evaluations of pathogen performance under potential environmental stresses viz. heat exposure and ultraviolet radiation. The studies reported here will examine the relevance of laboratory studies of these obviously crucial traits to field performances of several fungus isolates.

CHAPTER 2

THE ISOLATION OF *METARHIZIUM* SPECIES FROM SOIL, MORPHOLOGICAL CHARACTERIZATION, AND GENETIC IDENTIFICATION

Abstract

The entomopathogenic *Metarhizium* spp. are among the most commonly used fungi for biological control of pest insects in the field. The anamorphic stage of this soil-inhabiting group of fungi is very important for several reasons, not the least of which is their ability to infect a wide range of arthropods. Thousands of *Metarhizium* isolates have been acquired, and they display a wide assortment of characteristics, both morphologically and in insect-control effectiveness. The acquisition, identification and characterization of new isolates are basic-research activities, but these studies are crucial to development of *Metarhizium*-based biological control programs. The present study describes and compares eight new *Metarhizium* isolates plus two commercial isolates (ARSEF 1095 and 324) as to the following topics: the methods used to isolate them from soil, morphology-based isolate identification, and DNA-based identification. The last item was accomplished by Amplified Fragment Length Polymorphisms (AFLP) analysis, and nuclear ribosomal DNA sequence. Morphological differences were abundant between colony phenology, shape and color, as well as conidial size. But identification based exclusively on morphological traits is difficult and inconclusive, even for experts. Identification based on molecular methods was much more conclusive and illustrated important similarities better than did morphological traits. The morphologic and genetic

variations among isolates of the same species examined in this study suggest that new isolates should be subjected to in-depth characterization and evaluation of their physiological traits before being given serious consideration for use as pest-insect control agents.

1. Introduction

Metarhizium, a mitosporic soil-inhabiting fungus, is known to infect and cause disease in over 200 species of arthropods (Bidochka et al., 2001; Roberts and St. Leger, 2004). *Metarhizium* was first used as a microbial control agent against insects in 1879, when Elie Metchnikoff used it experimentally to control a pest grain beetle, *Anisoplia austriaca* in soil (Metschnikoff, 1880; Roberts and St. Leger, 2004). Since this time, *Metarhizium* has become one of the most commonly used fungi for biological control of pest insects in the field.

While *Metarhizium* spp. have been observed in nature only as an asexual anamorph, the teleomorph phase, when found, belongs in the genus *Cordyceps* (Ascomycota). *Cordyceps* includes nearly 400 described species that are generally endoparasitoids, mainly on insects and other arthropods (Bidochka et al., 2001). *Metarhizium* isolates display the characteristics of clonal populations of fungi, viz. widespread occurrence of identical genotypes (Milgroom, 1996; Bidochka et al., 2001). However, they are also thought to have the potential for parasexual reproduction (Tinline and Noviello, 1971; Bidochka et al., 2001), possibly occurring by the fusion of protoplasts within insects simultaneously infected by two or more compatible isolates (Pendland et al., 1993), although this has yet to be validated in nature.

New isolates of *Metarhizium* are obtained from two sources: either naturally infected insects, or soil (Milner, 1992; Vanninen, 1996; Shah et al., 1997). Generally, it is thought that fungi collected from a dead insect will be more virulent and better adapted for control of that pest-insect species in that habitat than isolates collected from non-insect substrates (Bidochka et al., 2001). Unfortunately, it usually is difficult to acquire new isolates directly from insect cadavers because field-killed insects are promptly removed by scavengers. Live naturally infected insects are a difficult source of new fungi since the insect must be collected, transported, and maintained in a laboratory, often by the thousands and for several days, to yield results (Shah et al., 1997). There are various methods to extract *Metarhizium* from soil. Two common methods are insect baiting, and using selective media (Goettel and Inglis, 1997). **Insect baiting** involves placing live insects of a species known to be susceptible to entomopathogenic fungi in a container of field-collected soil, and watching for mortality and mycosis. **Selective media** for *Metarhizium* usually will include an antibacterial agent (e.g., gentomycin), and a minute amount of a fungicide (e.g., dodine) to which entomopathogenic fungi tend to be more resistant than non-entomopathogenic fungi (Goettel and Inglis, 1997).

The pursuit of effective new fungal biological control agents for insect control includes not only obtaining new isolates, but also the identification to species of those isolates. Phenotypic traits expressed by particular species may aid in the selection process. For example, most *Metarhizium* spp. are pathogenic to a wide range of insects, but *M. acridum* is much more host specific, and in general is infectious only to orthopteran insects (Bridge et al., 1997; Goettel and Jaronski, 1997; Peveling and Demba,

1997; Driver et al., 2000; Milner et al., 2002; Alston et al., 2005). Identifying these entomopathogenic fungi to species based on morphological characteristics alone is extremely difficult due to a lack of sufficiently detailed morphology-based identification keys; and this approach may result in misidentifications (Fernandes et al., 2010). Therefore, DNA-based techniques for identifying *Metarhizium* spp. are now commonly used and provide accurate results (Driver et al., 2000; Entz et al., 2005; Bischoff et al., 2009).

Due to the current high level of interest in *Metarhizium* research and field use, and to the advancement of scientific technology, the taxonomy of *Metarhizium* spp. is being resolved. The recognition of several new species within the genus *Metarhizium* recently was described (Bischoff et al., 2009). In the new scheme, isolate identification is based on molecular studies, primarily DNA sequencing. The majority of extant *Metarhizium* spp. isolates have not yet been examined using molecular tools, but placement of most isolates is expected within the next few years.

This study describes the acquisition of eight new *Metarhizium* isolates from soil samples. These isolates were compared with each other and with two commercialized isolates obtained from a culture collection as to the following traits: (a) morphological characterization and (b) genetic identification by Amplified Fragment Length Polymorphisms (AFLP), nuclear ribosomal DNA sequencing.

2. Material and methods

2.1. Fungal isolates

Ten isolates of *Metarhizium* were used in this study, two of which (ARSEF 324 and ARSEF 1095) were received from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). Bischoff et al. (2009) identified ARSEF 324 as *M. acridum* and ARSEF 1095 as *M. brunneum*. The remaining eight specimens are cataloged in the Donald W. Roberts culture collection (DWR) (Utah State University, Logan, Utah). Fernandes et al. (unpublished) using the methodology of Bischoff et al. (2009), identified DWR 200, DWR 203, DWR 312, and DWR 313 as *M. guizhouense*; DWR 261 as *M. brunneum*; and DWR 338, DWR 346 and DWR 356 as *M. robertsii* (Table 2.1). All DWR isolates were obtained from soil samples by the procedure described below.

Soil samples were taken from the top 2 inches of soil from several locations in the Western United States, and placed individually in a sterile collection bag. The soil samples were transported to our Utah State University laboratory (Logan, Utah) and stored at 2°C until processed. Selective medium [(potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 0.1% yeast extract (Technical, Difco) (PDAY); 0.1% gentomycin sulfate (50 mg/ml), and 0.002% (active ingredient) Diodine)] was distributed into 95 × 15 mm Petri plates (23 ml/ plate). For each soil sample, 5 grams of dirt was mixed with 50 ml autoclaved distilled H₂O. For each suspension, 50 µl was spread with a glass spreader onto selective medium plates which were incubated at 28°C, and checked after 7, 14, and 21 days for colonies with

phenotypes commonly noted with colonies of entomopathogenic fungi. Transfers were made from these colonies, and the fungi were evaluated using a compound microscope, and monocultures were made from colonies with *Metarhizium*-like appearance (Tulloch, 1976; Barnett and Hunter, 1998; Driver et al., 2000) transferring to PDAY plates.

2.2. *Morphology of isolates*

All isolates spot were inoculated at least three times on PDAY plates using a 0.3-mm-wide hypodermic needle, and cultured for 20 days in the dark at 28°C. Colonies of each isolate were evaluated as to their general aspect, color of conidial masses, and colony reverses. The micromorphology of each isolate also was evaluated according to the microculture technique described by Rivalier and Seydel (1932). Briefly, PDAY medium was solidified in Petri plates and blocks cut and placed on sterile microscope slides. The blocks were inoculated with conidia, and incubated at 28°C high-humidity (lined with wet filter paper) dishes. Slides were stained with lactophenol cotton blue, covered with a cover-slip, and micro-morphologies were observed at 1000 × magnification. Furthermore, thirty conidia of each isolate were measured using the Vickers AEI Image Splitting Eyepiece measuring system (August Waeldin Inc., New Hyde Park, NY, USA) to determine average length and width of conidia.

2.3. *Amplified Fragment Length Polymorphism (AFLP) analysis*

Isolates were cultured in 250-ml flasks containing 50 ml of potato dextrose broth (Difco Laboratories, Sparks, MD, USA) (PDB). Broth cultures were shaken at 130 rpm at 25°C for 5 d. The fungal mycelia were collected by vacuum filtration onto Whatman

#1 filter paper (Whatman International Ltd., Maidstone, Kent, UK) and stored at -20°C . The mycelia were ground in liquid nitrogen with a mortar and pestle, suspended in 1 ml sterile TE buffer (Tris-EDTA: 10 mM Tris; 0.1 mM EDTA, pH 8.0) and stored at -20°C until DNA extraction. After defrosting, 70 mg of each isolate was extracted using the DNeasy[®] Plant Mini Kit (Qiagen Sciences, Maryland, MD, USA), following the manufacturers protocol. DNA extracts were electrophoresed on 0.7% agarose gels at 120 V in Tris-Acetate-EDTA buffer to estimate DNA quality and quantity. The gels were stained with ethidium bromide (Sigma, St. Louis, MO, USA) and visualized under ultraviolet irradiation.

All samples were genotyped using a modification of the Amplified Fragment Length Polymorphism protocol of Vos et al. (1995). Three out of ten isolates were subjected to the protocol in duplicate for the purpose of evaluating reproducibility of resolved AFLP banding patterns. The DNA samples (ca. 50 to 100 ng) were double digested for 1 h at 37°C with 5 units EcoRI and 5 units MseI, 5 μl 10 \times RL buffer (100 mM Tris-Hac; 100 mM MgAc; 500 mM KAc; 50 mM DTT, pH 7.5; stored at -20°C), and sterile double-distilled water up to 50 μl . After digestion, ligation was carried out by adding 10 μl of a solution containing 5 pmol EcoRI-adapters and 50 pmol MseI-adapters, 1 unit T4 DNA-ligase, 1.2 mM ATP, 1 μl 10 \times RL buffer, and double distilled water up to 10 μl . The incubation was continued for an additional 3 h at 37°C , and the reaction mixture then stored at -20°C .

A PCR pre-amplification was carried out containing 6 μl ligated DNA, 5 pmol Eco+A, 5 pmol Mse+A, 0.2 mM dNTP, 1.5 mM MgCl_2 , 1 unit *Taq* polymerase, 5 μl 10 \times

PCR buffer, and sterile double distilled water up to 50 μ l. The PCR pre-amplification included one initial cycle of denaturation at 72°C for 2 min, followed by 29 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min in a Thermocycler GeneAmp[®] PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Ten microliters of pre-amplification products with 2 μ l of loading dye were electrophoresed on 1.4% agarose gels at 120 V in Tris-acetate EDTA buffer, stained with ethidium bromide and visualized under ultraviolet irradiation to confirm the presence of pre-amplification products.

Multilocus AFLP profiles were obtained from three additional PCR amplifications. Preliminary examinations of AFLP banding patterns revealed very few markers when the conventional AFLP primers plus three selective nucleotides were used. Consequently, in order to increase the number of scorable markers per PCR reaction, only a single selective nucleotide was used in conjunction with the MseI primers at this stage. Overall, we used three different primer combinations for the second selective PCR (the actual DNA fingerprint-generation stage): MseI+A and EcoRI+AGC, MseI+A and EcoRI+AGG, and MseI+A and EcoRI+ACC. For each primer combination, a PCR amplification was carried out containing 2.5 μ l PCR pre-amplification product diluted 1:10 in water, 0.5 pmol Eco primer, 2 pmol Mse primer, 0.2 mM dNTP, 1.5 mM MgCl₂, 0.5 unit *Taq* polymerase, 1 μ l 10 \times PCR buffer, and sterile double-distilled water up to 10 μ l. The PCR amplification was run under the following thermalcycler parameters: 94°C for 2 min, then start a ‘touchdown’ PCR procedure using a 30 sec denature step at 94°C, a 30 sec annealing step at 65°C, and a 1 min extension step at 72°C. This procedure was

repeated 9 times, each time reducing the annealing temperature by 1°C. The annealing temperature of the final ‘touchdown’ step was 57°C, followed by 30 cycles of the following conventional thermalcycler program: 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec.

A size standard (ABIs ROX 400) was added to each PCR product and loaded into an ABI Prism[®] 3100 Genetic Analyzer. AFLP markers between 50-400 bp were scored as to presence/absence characters with Genographer software (freely distributed at <http://hordeum.oscs.montana.edu/genographer/>). Cluster analysis was performed using NTSYS-pc Vers. 2.1 (Exeter Software, Setauket, NY, USA) and a dendrogram based on data from all three primer pairs was derived from the similarity matrix calculated using Dice Coefficient. Cluster analysis was performed using the unweighted pair-group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973).

2.4. Nuclear ribosomal DNA sequencing

The genomic region from the 3’ end of the 16S rDNA to the 5’ end of the 28S rDNA gene (which flanks the Internal Transcribed Spacer region, ITS1 and ITS2, and includes the 5.8S gene) was PCR amplified with a pair of general fungal primers: TW81 (5’-GTTTCCGTAGGTGAACCTGC-3’) and AB28 (5’-ATATGCTTAAGTTCAGCG GGT-3’) (Curran et al., 1994; Entz et al., 2005), forward and reverse primers, respectively. The PCR reaction mixture consisted of 50 ng DNA, a final concentration of 50 pmol of each primer, 0.6 mM dNTP, 2.5 mM MgCl₂, 1 unit *Taq* polymerase, 1× PCR buffer, and sterile double-distilled water up to 50 µl. The PCR amplification program was based on one initial cycle at 95°C for 5min, followed by 35 cycles of denaturation

step at 95°C for 1 min, annealing step at 50°C for 45s, and extension step at 72°C for 2 min, and an additional 10 min of extension step at 72°C.

Five microliters of ITS-PCR products with 2 µl of loading dye were electrophoresed at 120 V on 1% agarose gel using Tris-acetate EDTA buffer. A 100 bp DNA ladder (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) was run on the same gel as the size marker. After electrophoresis, the gels were stained with ethidium bromide and visualized under ultraviolet irradiation.

The ITS-PCR amplified fragments from the 10 *Metarhizium* spp. isolates were purified using a Montage[®] PCR centrifugal filter device (Millipore Corporation, Bedford, MA, USA). All ITS-fragments were sequenced directly on an ABI Prism[®] 3100. The sequences acquired in this study were aligned with the Seqman program (DNASTar Inc., Madison, WI, USA). A neighbor-joining dendrogram was produced using MEGA Vers. 3.1 (Kumar et al., 2004) based on the Jukes-Cantor model of nucleotide substitution.

2.5 Statistical analysis

Variation in conidial size (width x length) among isolates was assessed using a one-way ANOVA. Assumptions of normality and homogeneity were met without transformations. The assumptions of normality and homogeneity of variance were met without transformations. All computations were done using SAS version 9.1.3. Conidia-size mean comparisons were assessed using an unadjusted family-wise Type I error rate to maximize power to detect potential differences; *P*-values less than 0.05 were considered as significant. Data analyses were generated using the GLM procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows.

3. Results

3.1. Soil screening

Soil samples were collected from nine states within the United States (Alaska, Arizona, Colorado, Idaho, Montana, Nevada, New Mexico, Utah, and Wyoming). The total number of soil samples collected was 474; eight (1.6%) of these samples afforded new isolates of *Metarhizium*. Five of the new isolates were from Arizona soil (DWR 200, DWR 203, DWR 313, DWR 338, and DWR 346); two isolates from Utah soil (DWR 312 and DWR 356); and one isolate from Alaska soil (DWR 261) (Tab 2.1).

3.2. Morphology of isolates

Conidial width and length varied significantly between isolates. The greatest average width was 5.4µm (DWR 313) and the smallest averaged width was 2.7µm (ARSEF 1095). These isolates, DWR 313 and ARSEF 1095, also had the longest and shortest average length; 9.2 and 5.9µm, respectively. Generally, conidial length and width was consistent among isolates (i.e., long and wide or short and thin), although some isolates varied, being long and thin (DWR 261), or wide and short (DWR 312). Conidia size comparisons were made using an average of length x width for each measured spore. DWR 313 was significantly larger than all other isolates, while ARSEF 1095 and DWR 346 were statistically smaller than all other isolate spores (Fig. 2.1). The spore size of ARSEF 324, the only *M. acridum* included in this study, was not statistically distinct from many the other *Metarhizium* spp. isolates, having an average length of 8.35µm and width of 4.25µm.

Morphological observations suggest phenotypic variability and similarity among many of the isolates (Fig. 2.2). The coloration of a ARSEF 324 colony is much lighter green than any other isolate, while DWR 261 produced large amount of mycelium. DWR 356, 346, 338, 203, and ARSEF 1095 produced conidia that were a dark green/brown color. DWR 200, 312, and 313 had dark green conidia and uneven edge growth. A radial sulcate growth pattern was consistently obvious in ARSEF 324 colonies, and slightly observable with ARSEF 1095.

3.3. DNA analyses

All the new isolates acquired in this study were identified as *M. anisopliae* s.l. by both methods of molecular analysis. This study was done prior to the Bischoff et al. (2009) publication and used different methodology; however, when the Bischoff et al. DNA-extension factor method was used later to identify these isolates to species, the AFLP groupings matched the new names. The sequenced rDNA fragment lengths were very uniform among isolates, after accounting for alignment gaps. The final sequence alignment was 455 bp, and contained 30 variable nucleotide sites. A neighbor-joining dendrogram of the rDNA sequencing showed the ten isolates clustering in to 4 groups (fig 2.3). ARSEF 324 was the most separated from all other isolates. DWR 261 and ARSEF 1095 were highly similar in the region sequenced, these we also separated from the other isolates but with a low confidence interval (24%). DWR 356, 346, and 338, being somewhat similar to each other, formed the third group, and were separated from the remaining fourth group by a confidence level of 64%. The fourth group was comprised of DWR 200, 313, 203, and 312, all being closely related.

AFLP analyses using the three primer pairs produced 205 polymorphic loci. Replicate isolates scored a 0% error rate, confirming reproducibility of the results. The UPGMA-dendrogram-encompassing variation among all isolates confirmed the clustering observed in the rDNA sequencing. Considerable genotypic variability was observed, especially between the *M. acridum* isolate and the other *Metarhizium* spp. isolates (Fig. 2.4). DWR 203 and 313 were nearly 100% similar, as was DWR 346 and 356.

4. Discussion

Isolating *Metarhizium* from soil can be challenging due to the abundance of co-inhabiting soil microorganisms. Nutrient competition and the use of dodine likely contributed to the low percentage of isolated colonies. Furthermore, soil samples were mostly taken from dry, temperate areas of the western USA which are not ideal conditions for *Metarhizium* growth. In contrast, 228 isolates of insect-pathogenic fungi were obtained from 590 soil samples from Finland using an insect-baiting method (Vanninen, 1996). None of the new isolates acquired in this study were identified as *M. acridum*; the likelihood of finding *M. acridum* was low reflecting that it has rarely been isolated from the Western hemisphere (Barrientos-Lozano et al., 2002), and is known to be more sensitive than other *Metarhizium* spp. isolates to the fungicide dodine (Rangel et al., unpublished).

Traditional identification of *Metarhizium* spp. is based on morphological features of colonies produced on artificial media, and microscopic examination of spores, conidiophores, and other structures (Bridge et al., 1997). This can be difficult and often

results in misidentifications even by experts familiar with *Metarhizium* (Fernandes et al., 2010). Previous identification keys described *M. anisopliae*, a group to which all the isolates of this study would have belonged before 2009, as having colonies in many shades of green, sepia or isabelline; the conidia are cylindrical to oval, often slightly narrowed in the middle, usually truncate at both ends, and can measure 3.5 – 9.0 μm in length, usually 5.0 – 8.0 μm (Tulloch, 1976). *M. acridum* isolates, however, produce dark yellow-green conidial masses, and conidia typically are ovoid, and measure 4.5 (± 0.41) in length \times 2.6 (± 0.6) in width (Driver et al., 2000). Conidial measurements observed in this study did not distinguish ARSEF 324 from the other isolates (see Fig. 2.1). Lomer et al. (2001) also noted that *M. acridum* is indistinguishable from other *M. anisopliae* varieties on the basis of spore size and shape.

Phenotypic characteristics (viz., conidia color, colony shape and size), seem to be correlated with genotype; and they may help in categorizing new *Metarhizium* isolates as either *M. acridum* or not — although, in no way should this be considered conclusive. ARSEF 324, the *M. acridum* isolate included in this study, had light green conidia, floccose colony texture, and produced deep radial sulcations in its colonies. All the other *Metarhizium* isolates had darker green conidia (some near brown), and generally did not express heavy radial sulcations. Unfortunately, morphology is not decisive for species identification of the *M. anisopliae* complex, as an individual isolate will sometimes exhibit different morphological aspects under varying environmental and physiological conditions (Entz et al., 2005).

AFLP and rDNA sequencing analyses clearly distinguished ARSEF 324 from other isolates (see Figs. 2.3 and 2.4). The primers for the rDNA sequencing used in this study are specific to the highly conserved ITS1, ITS2 and 5.8S region of the rDNA, making it an excellent procedure for identifying isolates of different species. With isolates that are closely related, which is common in anamorphic clonal fungi like *Metarhizium*, rDNA sequencing does not illuminate the strain differences. AFLP sequencing is non-specific and generally useful in demonstrating variability between closely related isolates, but less adept at categorizing isolates of different species. Using both methods in this study helped to recognize the relatedness of the isolates as well as their variability.

The clonalism is further illustrated with DWR 346 and DWR 356; soil isolates collected nearly 700 km from each other. These isolates are extremely similar both morphologically and genetically. Alternatively, genetic relatedness does not seem to always extend to morphological similarities. In the case of ARSEF 1095, isolated from Austria, and DWR 261, an Alaskan isolate, the AFLP results indicate that they have about 98% similarity, while morphologically, they were among the isolates most distinct from each other, e.g. their conidial size is significantly different. They do have in common, however, that they are the two most northerly collected isolates in this study.

Another example of this unpredictable correlation between morphology and genetic relatedness is seen in the cluster of Arizona isolates, DWR 200, 203, 313. The AFLP analysis indicated they have about 98% common characters. Morphologically, DWR 200 and 313 were very similar in colony appearance and shape, while DWR 203

was quite different. On the contrary, DWR 200 and 203 had similarly sized conidia, while DWR 313 was significantly larger.

The results of this study were similar to those of other studies and demonstrate the potential morphological variability found in the *M. anisopliae* complex (Fernandes et al., 2010). Morphological characteristics can potentially influence field efficacy as noted in studies involving conidial pigmentation and susceptibility to ultraviolet radiation (Braga et al., 2006; Rangel et al., 2006b). In addition, this study illustrates the difficulty of identifying relatedness based primarily on morphological characteristics, and the usefulness of DNA sequencing as a tool for better identification and classification of *Metarhizium* spp. The variation morphologically and genetically among isolates of the same species observed in this study is a further indication that new isolates must be subjected to in-depth evaluations of characteristics before being considered for use as pest-insect control agents.

Table 2.1 *Metarhizium* spp. isolates. Culture collection identification number, source of isolation material, origin of acquisition, coordinates (GPS coords: deg, min) of acquisition, colony description after 20 days growth on PDAY media, and average dimensions of conidia (length \times width).

Isolate	Host	Origin	Coordinates		Conidia Size
<i>Metarhizium acridum</i>					
ARSEF 324	Orthoptera	Australia	~S 19°42.219	~E 145°46.264	8.36 ±0.16 x 4.26 ±0.08 μm
<i>Metarhizium brunneum</i>					
ARSEF 1095	Lepidoptera	Austria	~N 47°30.584	~E 14°33.02	5.92 ±0.22 x 2.70 ±0.07μm
DWR 261	Soil	Talkeetna AK, USA	N 62° 08.906	W 150° 02.303	8.52 ±0.22 x 3.51 ±0.04 μm
<i>Metarhizium guizhouense</i>					
DWR 200	Soil	Snowflake AZ, USA	N 34° 42.924	W 110° 01.860	8.00 ±0.14 x 4.30 ±0.07 μm
DWR 203	Soil	Winslow AZ USA	N 35° 03.225	W 110° 35.388	7.50 ±0.14 x 4.30 ±0.05 μm
DWR 312	Soil	Logan UT, USA	N 41°46.465	W 111° 46.248	7.85 ±0.14 x 4.49 ±0.06 μm
DWR 313	Soil	Sedona AZ, USA	N 34° 57.271	W 111° 45.148	9.22 ±0.13 x 5.4 ±0.13 μm
<i>Metarhizium robertsii</i>					
DWR 338	Soil	Jacob Lake AZ, USA	N 36° 43.555	W 112° 07.522	7.76 ±0.17 x 4.08 ±0.06 μm
DWR 346	Soil	Snowflake AZ, USA	N 34° 41.222	W 110° 06.199	6.51 ±0.15 x 2.77 ±0.10 μm
DWR 356	Soil	Flaming Gorge UT, USA	N 40° 51.225	W 109° 33.964	7.41 ±0.15 x 3.48 ±0.04 μm

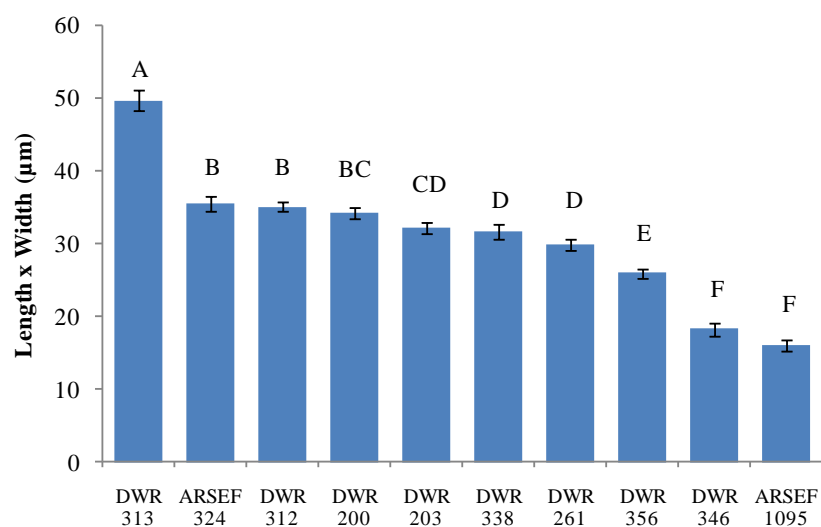
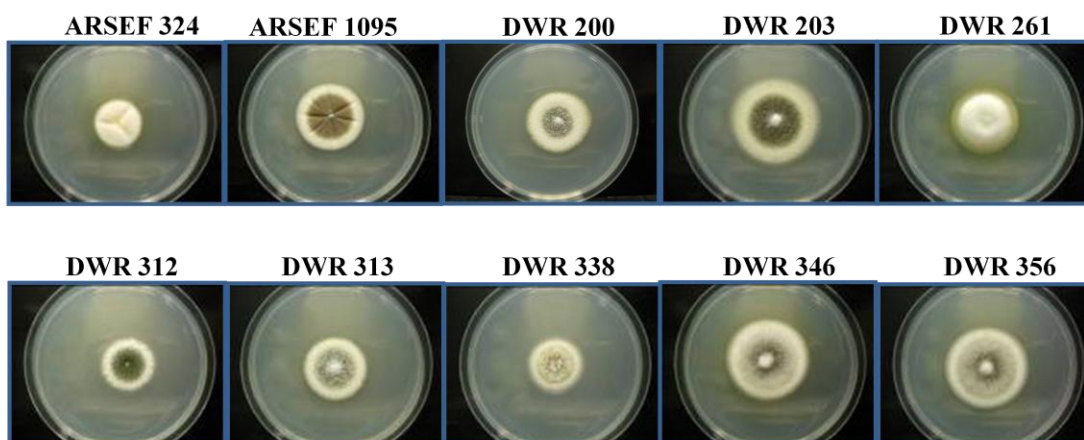


Figure 2.1 Average length-by-width measurements of conidia ranked largest to smallest. Letters represent statistical differences computed using PROC GLM in SAS 9.1.1 using the LSD method for mean variance comparisons.

8 Days growth



20 Days growth

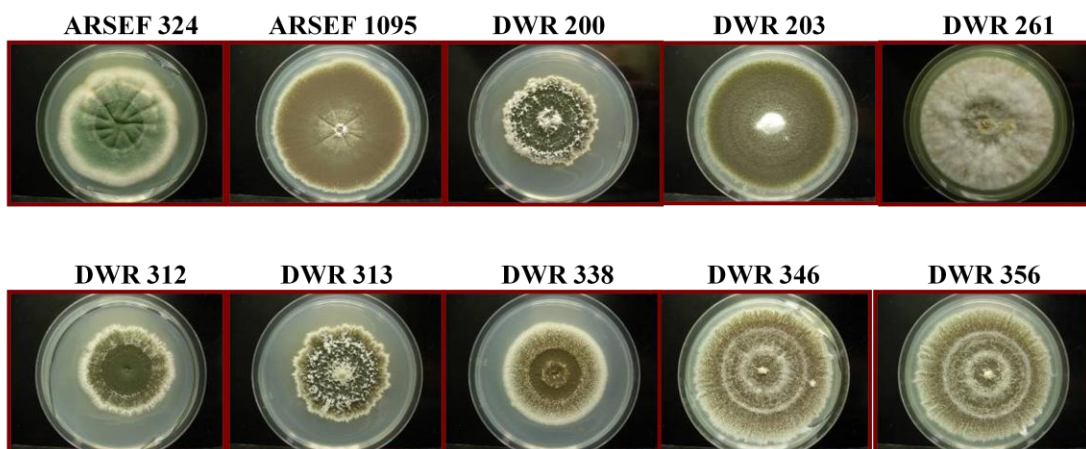


Figure 2.2 Colonies of *Metarhizium* spp. isolates after eight and 20 days growth on PDAY medium at $28\pm 1^\circ\text{C}$.

rDNA Sequencing

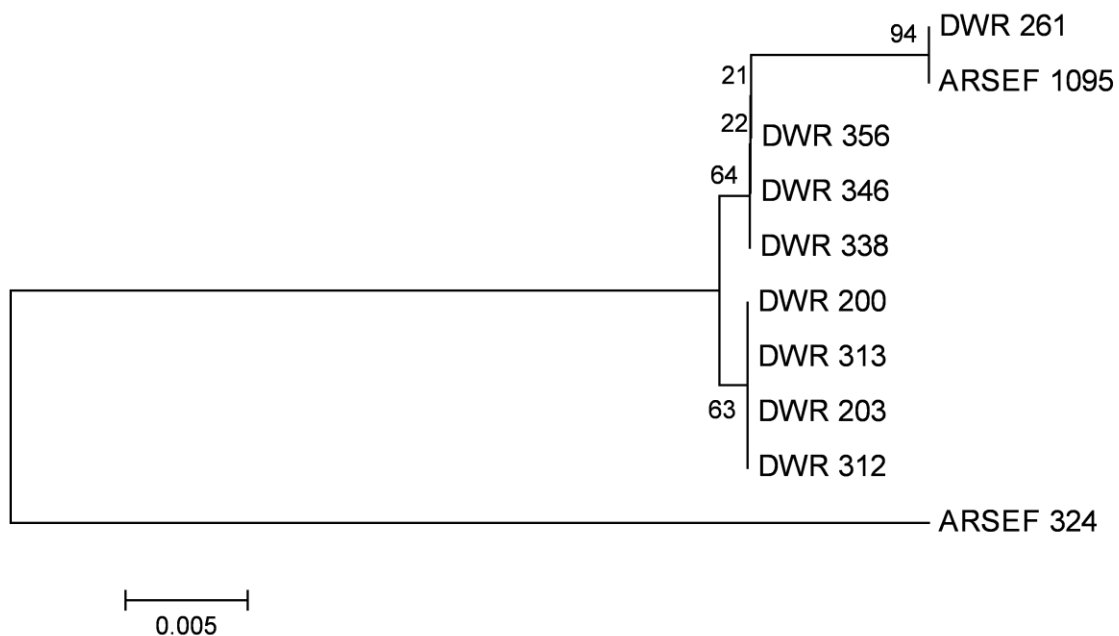


Figure 2.3 Neighbor-joining dendrogram generated with rDNA sequence data of 10 *Metarhizium* spp. isolates.

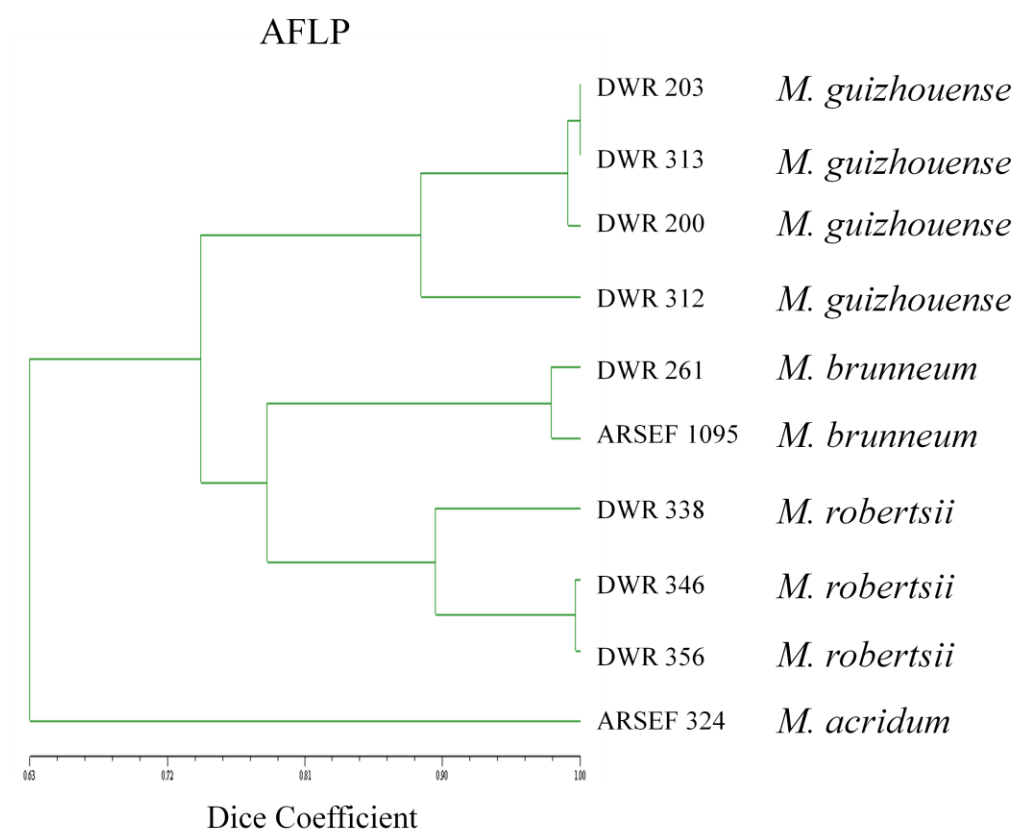


Figure 2.4 AFLP analysis using Dice coefficient generated by NTSYS-pc Version 2.1 for 10 *Metarhizium* spp. isolates.

CHAPTER 3
VIRULENCE OF TEN *METARHIZIUM* FUNGAL ISOLATES TO THE PEST
INSECT *ANABRUS SIMPLEX*

Abstract

The Mormon cricket is an orthopteran insect common in the western United States, and has been deemed an important agricultural pest for 150 years. Current methods for controlling Mormon crickets are primarily broad-spectrum chemical pesticides which, while effective, also raise many ecological concerns. Employment of a host-specific biological control agent, at least in at risk habitats, could obviate many of the disadvantages associated with non-specific chemical control methods. *Metarhizium*, an entomopathogenic fungus used for grasshopper and locust control in Africa and Australia, has shown promise for Mormon cricket control. This study assayed the virulence of ten *Metarhizium* isolates towards the Mormon cricket in the laboratory. Six of the ten isolates performed in an acceptable manner, causing high mortality by day five; while the four others were significantly less virulent. ARSEF 324, the only *M. acridum* isolate included in this study, was not the highly virulent isolates. All isolates tested were pathogenic to the Mormon cricket.

1. Introduction

Settlers endeavoring to scratch out a living west of the Rocky Mountains made their first serious attempt at agriculture in Utah in 1848. Their hardships were increased by an outbreak of *Anabrus simplex*, an orthopteran pest commonly known today as the

Mormon cricket (MC). Most Utah residents are familiar with what is known as the “Miracle of the Seagulls, ” wherein local populations of seagulls rescued their first-year crops by greatly reducing an overwhelming MC infestation (Ludlow, 1992). Despite this temporary respite, more than 150 years later this insect continues to cause serious agricultural losses in Utah and is considered an important agricultural pest (UDAF, 2005).

The MC, actually a katydid more closely related to grasshoppers than true crickets, is a large, flightless insect with jumping legs and the ability to migrate many miles during its lifetime of up to 8 months (Gurney, 1939). It can weigh up to 6g (0.2 oz.) and measure as much as 60 mm (2 in.) in length (MacVean, 1990; Pfadt, 1994; Gwynne, 2001; Simpson et al., 2006). MC populations consist basically of one species, *A. simplex* (Bailey et al., 2005; Lorch et al., 2005). Generally, MC occur in low numbers and cause relatively insignificant damage to crops; but occasionally they can reach outbreak population levels which may last up to 15 years and cause devastating crop loss (Pfadt, 1994; Capinera et al., 2004).

Early MC control was often experimental and drastic. Examples include the use of fire, loud noises, toxic arsenic-based pesticides, and elaborate barrier systems to corral oncoming bands into oil or water pits (Gwynne, 2001). Current methods for controlling MC are generally based on broad-spectrum chemical pesticides. In the United States, carbaryl and malathion in wheat-bran baits and dimilin in a spray are the most commonly employed chemicals. These chemicals are effective at suppressing pest populations, but have the disadvantage that they are prohibited in many areas, such as near waterways and

habitats of threatened or endangered species. In addition, organic farmers and ranchers are not allowed to apply chemical products to their crops or rangelands for control of pest insects. Furthermore, broad-spectrum insecticides often will affect non-target beneficial insects, including: pollinators, resource producers (honey, silk or wax), natural enemies of insect pests, wildlife food, and scavengers. These deleterious effects on the environment are a major concern, and may, in some cases, cause greater ecological and economical damage than an uncontrolled pest.

Employment of a host-specific biological control agent, at least in at risk habitats, could obviate many of the disadvantages associated with non-specific chemical control. The insect pathogen *Metarhizium* is among the most promising biological control agents for Orthoptera pests. Several species of *Metarhizium* are naturally occurring, cosmopolitan insect pathogen that, when assayed against grasshoppers, were consistently found to be more virulent than *Beauveria bassiana*, another common insect pathogen often used for biological control (Bateman et al., 1996; Lomer et al., 2001).

Infection mechanisms and mortality rates differ between strains and varieties of *Metarhizium* and their hosts. *In-vitro* assays performed in controlled environments are useful in evaluating and comparing the virulence of different isolates in order to exclude ineffective isolates from those with potential to control pest insects. Accordingly, this study assays the virulence of ten *Metarhizium* isolates towards MC in a controlled laboratory setting.

2. Materials and Methods

2.1 Fungal inoculum

Ten *Metarhizium* isolates were included in this study. Three isolates [one *M. acridum* (ARSEF 324), one *M. robertsii* (ARSEF 2575), and one *M. brunneum* (ARSEF 1095) (Bischoff et al., 2009)] were obtained from a from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (USDA Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). The remaining seven isolates were isolated from soil as described in Chapter two of this thesis; Fernandes et al. (unpublished) using the methods of Bischoff et al. (2009) identified DWR 200, DWR 203, DWR 312, and DWR 313 as *M. guizhouense*; and DWR 338, DWR 346 and DWR 356 as *M. robertsii* (Table 2.1). Stock cultures were maintained at 4°C in test-tubes slants of potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9.

Conidia used for assays were harvested from 14-day-old cultures grown on PDAY plates (polystyrene, Petri dishes, 95x 15 mm , Fisherbrand Pittsburg, PA, USA) incubated in the dark at 28±1°C. Harvested conidia were suspended in 40 ml of 0.01% Tween 80 solution in 50-ml tubes (Modified polystyrene, Corning, Coning, NY, USA). Each suspension was vortexed and conidial concentrations were estimated by haemocytometer counts. A 100 ml conidial suspension with a concentration of 1 x 10⁷ conidia ml⁻¹ was prepared for each isolate. Suspensions were used immediately for bioassay experimentation.

2.2 The insect

Mormon crickets, between nymphal instars 4 and 7, were collected from rangeland and surrounding areas in Box Elder County, UT. Collected MC were held for 24 hours at $24\pm 1^{\circ}\text{C}$ and fed organic spinach leaves. Healthy crickets were then transferred to individual 9-oz. clear plastic cups (Solo, Urbana, IL, USA), containing 25g autoclaved Industrial quartz sand (Unimin Corporation) and closed with sterilized fiberglass screen stretched across the top and fastened with a rubber band.

2.3 Virulence assay

MC were treated with either 0.01% Tween 80 (control) or conidial suspensions while in their individual containers. Treatments were applied by spraying suspensions (or control) with a Preval Portable Sprayer (Preval sprayer Division, Precision Valve Corporation, Yonkers, New York) until run-off, followed by adding a single organic spinach leaf and replacing the screen covering. Each treatment consisted of 30 individually caged insects. MC were maintained with high humidity at $24\pm 1^{\circ}\text{C}$ with 12 hours light/dark. Mortality was read daily, but results were based only on the reading five days after treatment. Testing of each isolate was repeated at least 3 times at different periods of the MC season. Due to the difficulty in collecting and maintaining massive insect populations, all isolates could not be treated simultaneously. In order to harmonize results, all bioassay trials included a standard strain, ARSEF 2575.

2.4 Statistical analyses

To remove effects not due to entomopathogen treatments, observed mortality was adjusted using Abbott's formula (1925). The effect of treatment on mortality was assessed using a one-way ANOVA with an unbalanced incomplete block design. Prior to analysis, the Abbott-adjusted mortality data were arcsine-square root transformed to better meet the assumptions of normality and homogeneity of variance. To address heterogeneity of variance that persisted despite the transformation, a heterogeneous variances model was used to partition residual variance into two groups (Littell et al., 2006). One group was comprised of isolates ARSEF 1095, DWR 203, DWR 312, and DWR 338 for which adjusted mortality values were close to 100% and variances thus were small; the second group was comprised of isolates ARSEF 324, ARSEF 2575, DWR 200, DWR 313, DWR 346, and DWR 356 for which adjusted mortality values were typically smaller and thus variances were larger. Mean comparisons among treatments were unadjusted for family-wise Type I error to maximize power to detect potential differences; *P*-values less than 0.05 were considered as significant. Data analyses were generated using the MIXED procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows.

3. Results

While differences in mortality rates were observed, all test isolates of *Metarhizium* were pathogenic towards *A. simplex* to some degree. After 5 days exposure to conidial formulations, isolates ARSEF 1095, DWR 203 DWR 338, DWR 312, ARSEF 2575 and DWR 346 caused more than 95% mortality in MC (Figure 3.1). DWR 356,

caused 69.9 % adjusted mortality in treated MC, differed significantly from ARSEF 1095, DWR 203 and DWR 338. DWR 313, ARSEF 324 and DWR 200 infected and caused mortality in 59.9, 41.3, and 33.8% treated insects. Due to high production of mycelium causing difficulty in the application process, DWR 261 was not assayed with MC. After 7 days treatment all isolates except ARSEF 324 caused mortality in 90% or more treated insects, while ARSEF 324 reached an average of 84.4% mortality.

4. Discussion

The variation five days after inoculation was low, the majority of isolates caused above 70% mortality, and only two isolates were below 50% (DWR 200 and ARSEF 324). Distinctions between isolates based on mortality can be unclear. For this reason, lethal dose rather than lethal time, is often used to measure virulence (Blanford and Thomas, 2001; Faria et al., 2002; Ansari et al., 2004; Murad et al., 2006). The difficulty in using a dose method to compare isolates is that more insects and treatments are required for each assay. MC laboratory colonies have not been established to date, resulting in assays having to rely on the availability of field-collected crickets.

M. acridum isolates are known to specialize on orthopteran pests (Bridge et al., 1997; Goettel and Jaronski, 1997; Peveling and Demba, 1997; Milner et al., 2002; Alston et al., 2005), which makes these isolates the preferred choice for grasshopper and MC control in delicate environments where broad-host-spectrum isolates that may kill non-target organisms are inappropriate. Commercial programs utilizing *M. acridum* already exist in Africa, and Australia (Hunter et al., 2001; Arthurs et al., 2003; Milner et al., 2003; Ouedraogo et al., 2003). The fact that ARSEF 324, the only *M. acridum* isolate

tested in this study, was one of the slower killers does not eliminate it from consideration due to this isolate's advantage of host specificity to grasshoppers and closely related insects, and to its exceptional heat tolerance (Fernandes et al., 2010). Hunter et al. (2001) observed greater than 90% mortality after 14 days aerial treatment with FI-985 (ARSEF 324) in Australian plague locust field trial. Kershaw et al. (1999) suggested that there are at least two types of virulence strategies employed by *Metarhizium*, a "toxin strategy" and a "growth strategy". They also observed that *M. acridum* isolates generally produce little or no destruxins, a cyclo-depsipeptide fungal toxin that interferes with a host's calcium channel function. This lack of the toxin is an indication that *M. acridum* isolate prefer the slower "growth strategy" to kill a host. Moon et al. (2008) also found that ARSEF 324 produced very low levels of destruxins. In our assays, ARSEF 324 killed more than 80% of treated insects by day 7, indicating that it is pathogenic towards MC; but it takes longer than most non-*M. acridum* isolates to infect and kill. Roberts and St. Leger (2004) note that specialist strains generally produce little or no toxins as they have evolved a relationship that involves extensive growing within the host. Furthermore, high laboratory-bioassay mortality does not always translate into high levels of pest control in the field (Sosa-Gomez and Moscardi, 1998; Roberts and St. Leger, 2004).

Based exclusively on this study one would conclude that six of the ten isolates (ARSEF 1095, DWR 203 DWR 338, DWR 312 ARSEF 2575 or DWR 346) tested may be suitable for field use to control MC outbreaks. It is without question that an isolate's pathogenicity to a target insect is required before attempting confirmation in the field, but

virulence assays based on time-to-kill should not be the only selection tool used for distinguishing “good” from “inadequate” isolates.

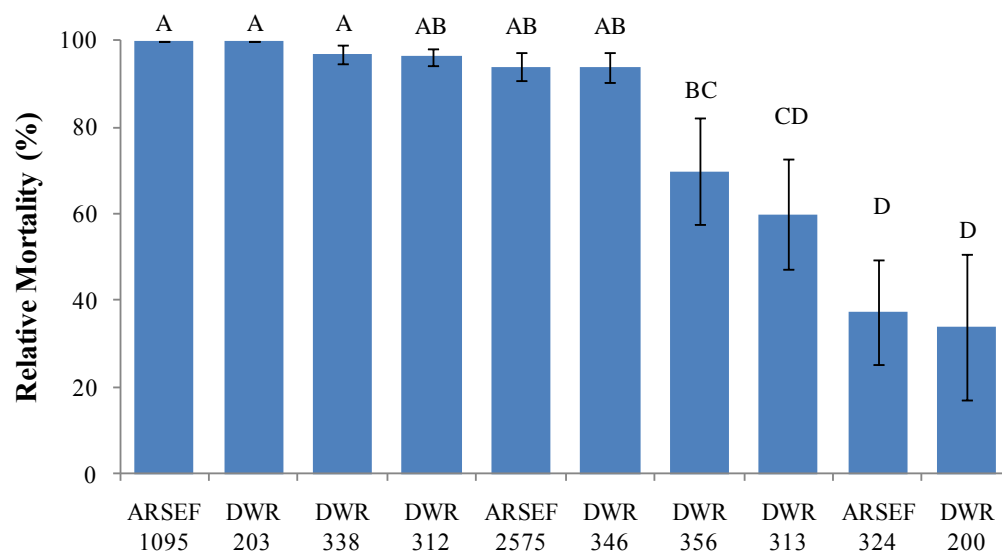


Figure 3.1 Abbott-adjusted mortality of Mormon cricket nymphs treated with one of ten *Metarhizium* spp. isolates five days after treatment, with standard error bars. Different letters represent statistically significant differences (P -values less than 0.05).

CHAPTER 4

MASS SPORE PRODUCTION CAPABILITIES OF TEN *METARHIZIUM* SPP. ISOLATES ON AN INEXPENSIVE SOLID SUBSTRATE MEDIA

Abstract

Metarhizium conidiospores are more environmentally persistent when mass produced by solid substrate fermentation in comparison to liquid fermentation. Individual strains of *Metarhizium* have unique spore production capabilities, so selecting an isolate with the capacity to produce sufficient spores using an inexpensive method is crucial to large-scale commercial programs. This study evaluates the spore production capabilities of ten *Metarhizium* spp. isolates on flaked barley, using a standardized method of solid substrate fermentation. DWR 338 produced the most spores (1.1×10^9 per 1 gram barley), closely followed by ARSEF 324 (1.0×10^9), DWR 346 (9.7×10^8), while DWR 356 (7.2×10^8); while DWR 200 produced the least amount of spores (5.5×10^7), along with DWR 203 (5.8×10^7), ARSEF 1095 (3.0×10^8) and DWR 261 (3.2×10^8) per gram of substrate. The viability of the harvested spores ranged from 90.75% (DWR 356) to 52.1% (DWR 200). There was only minor variability among the majority of isolates with regard to viability. The median viability was 74.09% (e.g., DWR 338). Based on production quantities and viabilities, the best isolates for field use of those tested in this thesis are: DWR 338, ARSEF 324, DWR 346 and DWR 356. The range of production capabilities was vast, ranging from 0.64 grams/ 100g barley flakes (DWR 203) to 8.56g (ARSEF 324). This indicates that production capabilities vary between fungi, and these differences cannot be ignored in selecting isolates for commercialization.

Also, many different factors are involved in the production of conidia. Modifying a standardized production method to better fit the needs of specific fungal isolates will likely enhance quality and quantity of spores produced.

1. Introduction

Two of the chief difficulties in developing fungal biological control agents for insect control are the delicate nature of large-scale (mass) production of conidia, and in successfully attaining long-term storage. Chemicals are often favored because they are inexpensive to mass produce and have a long shelf-life. The ability to grow large amounts of viable conidia on a low-cost substrate is paramount to the success of a commercialized fungal product (Goettel and Roberts, 1992). Fortunately, many isolates of the entomopathogenic fungi *Metarhizium* and *Beauveria* are able to develop independent of their insect hosts, which makes large-scale production of them both technically and economically feasible (Feng et al., 1994; Grimm, 2001). Historically, the hyphomycete *Metarhizium* was the first fungus to be mass produced for the purpose of controlling an insect pest (Krassiltschik, 1888).

Liquid fermentation is more advanced than solid substrate fermentation, and it would be expected to be more cost efficient for industry. Nevertheless, with *Metarhizium* the most environmentally persistent stage is the conidiospores, which normally are not produced in submerged culture, and thus are generally more abundantly produced by solid substrate fermentation (Auld, 1992; Jenkins and Goettel, 1997; Lomer et al., 2001; Roberts and St. Leger, 2004). A commonly used approach is to develop mycelium in liquid (submerged) culture, then inoculate solid substrates with this mycelium for

production of conidia. The nutrient sources must provide adequately balanced levels of carbon, phosphorous and nitrogen (which normally is present in rice bran, wheat bran, and barley flake) (Dorta et al., 1990; Boas et al., 1996; Dorta et al., 1996; Cherry et al., 1999; Grimm, 2001; Lomer et al., 2001; Roberts and St. Leger, 2004). Apart from an appropriate nutrient source, growth requirements also include a delicate balance of appropriate temperature, pH, water activity, and gas exchange of CO₂ and O₂ (Roberts and St. Leger, 2004; Grace, 2005). Another challenge is maintaining a sterile growth environment which is crucial since *Metarhizium* is often out-grown or killed by other microorganisms (Lingg et al., 1981; Roberts and St. Leger, 2004). To accommodate these requirements, mass production is sometimes accomplished in autoclavable polypropylene bags fitted with some type of filtered gas exchange system (Mendonça, 1992; Cherry et al., 1999; Roberts and St. Leger, 2004; Grace, 2005).

After fermentative growth is complete, spores must be harvested. This step may be accomplished in several ways; for example, conidia and infested substrate can be milled and used as biocontrol applicant, or conidia can be removed from grain. Separating the spores from the growth substrate can be done in several ways: floating in a water bath (conidia float while rice grains tend to sink), using metal sieves to separate conidia from grain, or using a centrifugal commercial vacuum device to collect conidia (Microharvester™) (Roberts and St. Leger, 2004; Grace, 2005). The most important considerations are the quantity (grams conidia) and quality (percent viability) of the end product.

Most entomopathogenic-fungus research is directed toward developing a product for controlling pest insects. Mass-production capability is an important aspect of isolate evaluation for field potential. Accordingly, this study appraises the mass production potential of ten *Metarhizium* spp. isolates employing organic flaked barley as the fermentative substrate.

2. Materials and methods

2.1. Fungal isolates

Ten isolates of *Metarhizium* were used in this study, of which, two (ARSEF 324 and ARSEF 1095) were received from the USDA-ARS collection of Entomopathogenic fungal cultures (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). Bischoff et al. (2009) identified ARSEF 324 as *M. acridum* and ARSEF 1095 as *M. brunneum*. The remaining eight specimens are cataloged in the Donald W. Roberts culture collection (DWR) (Utah State University, Logan, Utah). Fernandes et al. (unpublished) identified DWR 200, DWR 203, DWR 312, and DWR 313 as *M. guizhouense*; DWR 261 as *M. brunneum*; and DWR 338, DWR 346 and DWR 356 as *M. robertsii*, following the methodology prescribed by Bischoff et al. (2009) (Table 1.1). Stock cultures were maintained at 4°C in test-tube slants of Potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9.

2.2 Solid-substrate fermentation

Solid-substrate fermentation was accomplished using a three step method as described by Julie Grace at a solid-substrate fermentation workshop presented at USDA/ARS/NPARL Sidney, Montana (2005).

Step 1: Fungal conidia were produced on 23 ml of Potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9 media in 95 x 15 mm polystyrene Petri Dishes grown at 28°C.

Step 2: Using conidia produced in step one, a blastospore culture was produced in a liquid medium. The media consisted per liter of water, 10 g glucose, 10 g yeast extract, 4 g K₂HPO₄, 2 g KH₂PO₄, 1 g NH₄NO₃, 0.2 g MgSO₄, 0.2 g KCl, 0.002 g FeCl₂, 0.002 g MnSO₄, and 0.002 g ZnSO₄ with 1 ml (10mg/ml) of gentomycin. The medium was autoclaved in an Erlenmeyer flask with a cotton plug in the mouth to allow air exchange, cooled, and inoculated with conidia produced in step one. Flasks were placed on a gyratory shaker (172 rpm) and allowed to grow for 5 days at 24°C.

Step 3: In a polypropylene autoclave bag (8 x12cm Fisherbrand), 100 g organic flaked barley (Shangri-La Health Food, Logan, Utah) were added along with 60ml distilled H₂O. Bags were closed with a 10cm cotton plug tied in place, and autoclaved for 30min at 121°C. Bags were allowed to cool, opened in a sterile area, and 30 ml of blastospore culture was added for each isolate. Inoculated bags were placed at 28±1°C, massaged daily and allowed to grow for 14 days.

2.3 Spore harvesting

After solid-substrate fermentation was completed, each fermentation bag was emptied into a double-paper lunch sack (Giant Size lunch bags, Kroger CO., Cincinnati, Ohio, USA); the opening was folded 3 times, closed with staples and dried for 5 days. After drying, conidia were separated from the substrate using a series of mesh sieves (#20 and #100, Fisher Scientific) placed in a mechanical shaker for 15 min. Spore product was weighed, and conidia-per-gram was determined using a hemacytometer.

2.4 Spore viability

Immediately after harvesting, a suspension was prepared by adding a small amount of conidia to 0.01% Tween. Twenty μ l of suspension was transferred on to PDAY medium with 0.002% Benomyl (active ingredient) (Hi-Yield Chemical Company, Bonham, TX) in 35 x 10 mm Petri plates. Plates were placed at 28°C and allowed to grow for 48h. For each plate, 300 conidia were observed for germination (germtube visible at 400 x magnification).

2.4 Statistical analysis

An analysis of variance of the mean grams of product produced and the number of conidia produced for each isolate was performed. Assumptions of normality and homogeneity were met without transformations for grams produced, number of spores produced data was square root transformed to meet assumptions of normality and homogeneity. The statistical model tested for heterogeneous variances among isolates. Mean comparisons among treatments were unadjusted for family-wise Type I error rate

to maximize power to detect potential differences; *P*-values less than 0.05 were considered as significant. Data analyses were generated using the MIXED procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows.

3. Results

A wide range of production capabilities were observed between the ten isolates. Color of spores also varied, ranging from pale green to a dark green or dark green/brown. DWR 200 and 203 conidia were pale green, while DWR 338, 346, 356, and ARSEF 1095 had similar shades of green green/brown, while ARSEF 324 conidia were a rich green color that was unique from other isolates (Figure 4.1).

The average weight of spores harvested from 100 gram of flaked barley varied significantly among the *Metarhizium* isolates ($F = 12.17$, $p = 0.0001$), ranging from 0.64 g (DWR 203) to 8.56g (ARSEF 324) (Figure 4.2). DWR 338 and ARSEF 324 had the greatest yield and their productivities were not significantly different from each other. DWR 200 and DWR 203 both produced an average of less than 1 g of spores.

The number of conidia per gram of spores was calculated for each isolate. Due to conidial size differences among the isolates, spores/g also varied (Table 4.1). The most spores per gram was 2.2×10^{10} (DWR 346) and the least amount was 7.1×10^9 (DWR 200).

Calculation of the total number of spores produced on one gram substrate was calculated, and was found to vary between the isolates ($F = 8.21$, $p = 0.0001$). DWR 338 produced the most spores 1.1×10^9 per gram barley, closely followed by and not significantly different from ARSEF 324 (1.0×10^9), DWR 346 (9.7×10^8) and DWR 356

(7.2×10^8) (Fig. 4.3). DWR 200 produced the fewest spores (5.5×10^7) along with DWR 203 (5.8×10^7), ARSEF 1095 (3.0×10^8) and DWR 261 (3.2×10^8).

The spore viability after harvest ranged from 90.75% (DWR 356) to 52.1% (DWR 200) (Figure 4.4). There was only minor variability among the majority of isolates with regard to viability. DWR 338 was considered medium viability at 74.1% (Table 4.1).

4. Discussion

The variability in production capabilities among the ten isolates was not unexpected. Different *Metarhizium* isolates have different requirements for growth; including nutrition, pH, water activity, temperature optima, light, aeration, and incubation periods (Arzumanov et al., 2005). This study tested only one set of growth conditions for all the isolates; but the results would likely have been different if the growth conditions were modified. When designing a growth regimen, focusing on promoting spore production rather than mycelium propagation is important, as spores will be the inoculating unit. Limited nutrient and stressful conditions often encourage fungi to produce conidia rather than mycelium (Kamp and Bidochka, 2002).

ARSEF 324 was one of the best conidia producers in this study, and this isolate has been reported previously to grow and sporulate well on a variety of media (Nelson et al., 1996; Milner, 1997). ARSEF 324 also had among the highest conidial viabilities immediately after harvest. Previous studies have demonstrated that the most successful field trials are done with conidia that are >80% viable (Lomer et al., 1997). This may be a concern for several of the other isolates tested in this study, as only two (DWR 356 and

ARSEF 324) met this criterion. Nonetheless, only three isolates were below 70% germination (see Fig. 4.4). It is likely that optimization of the production and harvesting methods could increase viability and quantity of the conidia produced (Hong et al., 1999; Dalla Santa et al., 2004; Kassa et al., 2004, 2008; Damir, 2006; Ye et al., 2006). For example, increasing air flow to DWR 203 during culture on flaked barley increased yield ~150% (data not shown).

The amount of conidia recommended to treat a hectare of land varies; Hunter et al. (2001) considered $3-4 \times 10^{12}$ conidia a moderate dose, and $1-2 \times 10^{12}$ a low, but effective, dose when treating locusts in Australia with ARSEF 324. Based on 1×10^{12} conidia per ha, the grams of conidia required to treat a hectare of land ranged from 45.45g (DWR 346) to 140.85g (DWR 200) (Table 4.2). The flaked barley needed to produce the necessary grams of conidia ranged from 0.87 kg (DWR 338) to 17.39 kg (DWR 200). The Idaho Grain Market Report for October 15, 2009 gave the market-value price of barley to be around \$0.11 (US) for 1kg. These prices make the material cost reasonable for many of the isolates. After completing one of the first successful field trials using *Metarhizium*, Krassiltschik (1888) claimed that the factory, labor and substrate were very inexpensive; but that time was the principal limiting factor in spore production. In addition, another difficulty is avoiding contamination, as sterilizing large amounts of substrate is not casually accomplished (Roberts and St. Leger, 2004). Spore production for this study was realized in 100 g of flaked barley in bags; a relatively small amount in comparison to what would be needed for serious field treatments. While some

scale-up procedures would be straight forward, additional new steps would be needed to avoid contamination, including longer autoclave time, and strict sterile methods.

Mass production of *Metarhizium* spp. seems to be more of an art than science. Many different factors are involved in the production of conidia. Grace (2005) notes that some strains of *Metarhizium* do not do well in solid substrate fermentation. Isolates with slow grow rates are more susceptible to contamination, while others produce too much heat during growth and need to be cooled. Also, some will produce “cement-like” mycelium that binds the substrate (barley) together, thus minimizing surface area and spore production. This was seen in DWR 203, a poor spore produce. The best isolates for field use based on production quantities and viability are: DWR 338, ARSEF 324, DWR 346 and DWR 356. Selecting an isolate with good production capabilities makes the transition from laboratory experimentation to commercial employment less complicated. However, the production method of a low-spore-producing isolate that has other traits or characteristics of interest could be optimized to increase quantity or quality of conidia, and therefore it should not be summarily eliminated from consideration based only on mass production capabilities.

Table 4.1 For ten isolates of *Metarhizium*: The average weight of spores harvested from 100 grams of flaked-barley substrate; the average number of spores in one gram of spore product; the average total number of spores produced on one gram of substrate; and average percent viability of each isolate's product

	DWR 200	DWR 203	DWR 261	DWR 312	DWR 313	DWR 338	DWR 346	DWR 356	ARSEFARSEF 324	ARSEFARSEF 1095
Product weight per 100g substrate	0.81	0.64	2.84	3.96	3.97	6.76	4.38	3.72	8.56	1.58
Spores per 1g product	7.1×10^9	1×10^{10}	1.1×10^{10}	8.7×10^9	8.4×10^9	1.7×10^{10}	2.2×10^{10}	1.9×10^{10}	1.2×10^{10}	1.7×10^{10}
Total spore produced on 1g substrate	5.5×10^7	5.8×10^7	3.2×10^8	3.7×10^8	3.3×10^8	1.1×10^8	9.7×10^8	7.2×10^8	1×10^8	3×10^8
Viability of spores (%)	52.10	62.34	74.24	73.01	66.04	74.10	78.11	90.72	85.66	79.02

Table 4.2 For ten isolates of *Metarhizium*: The grams of spores needed to provide the field dosage of either 1×10^{12} and 5×10^{12} spores per ha^{-1} , and the kilograms of substrate needed to produce those spores.

		DWR 200	DWR 203	DWR 261	DWR 312	DWR 313	DWR 338	DWR 346	DWR 356	ARSEF 324	ARSEF 1095
1×10^{12}	Grams of spores	140.85	100.00	90.91	114.94	119.05	58.82	45.45	52.63	83.33	58.82
	Kilograms of substrate	17.39	15.63	3.20	2.90	3.00	0.87	1.04	1.41	0.97	3.72
5×10^{12}	Grams of spores	704.23	500.00	454.55	574.71	595.24	294.12	227.27	263.16	416.67	294.12
	Kilograms of substrate	86.94	78.13	16.01	14.51	14.99	4.35	5.19	7.07	4.87	18.62

Harvested conidia



Figure 4.1 *Metarhizium* conidia grown on 100 grams of organic flaked barley for 14 days, dried for 5 days, and harvested using shaken sieves.

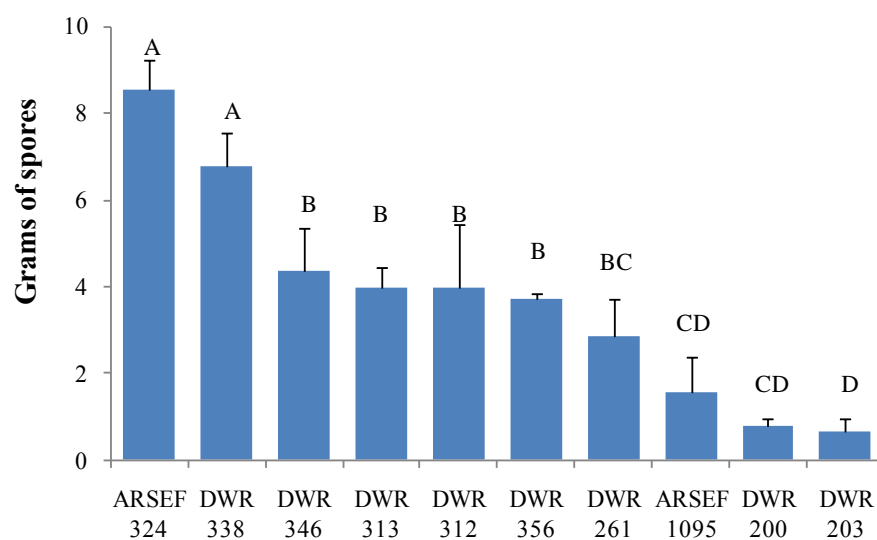


Figure 4.2 The average weight in grams of the harvested spores of ten *Metarhizium* spp. isolates produced on 100 grams of organic flaked barley. Bars represent standard errors of 3 repetitions. Statistically significant differences are presented by letter designations below the x-axis.

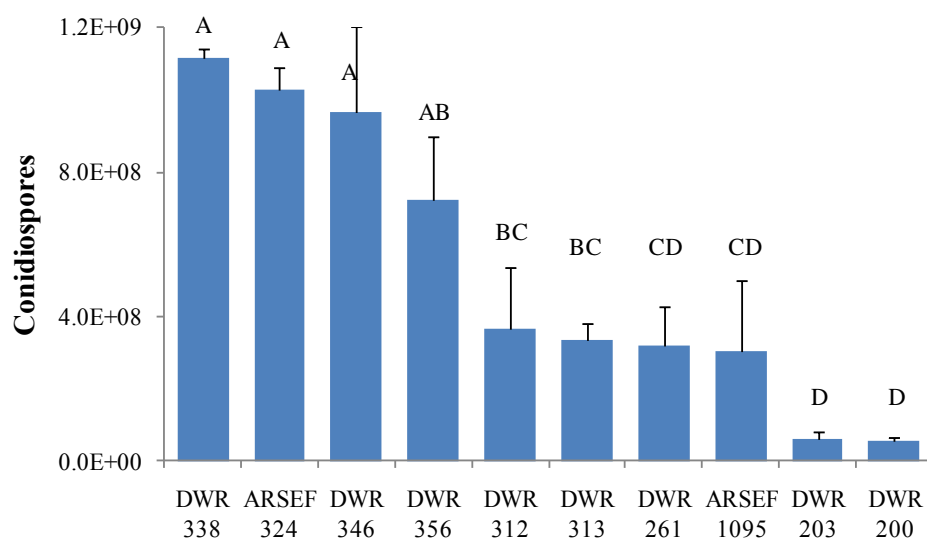


Figure 4.3 The average number of spores harvested from one gram of organic flaked barley for ten *Metarhizium* spp. isolates. Bars represent standard errors of 3 repetitions. Statistically significant differences are presented by letter designations below the x-axis.

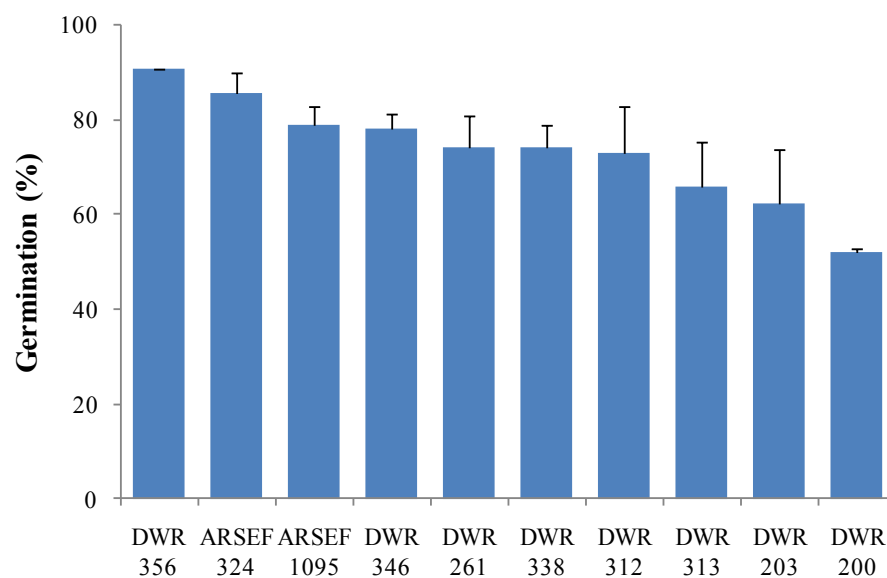


Figure 4.4 The average percent viability of the harvested spores from ten *Metarhizium* spp. isolates produced on 100 grams of organic flaked barley. Percentages were based on 300 spore observations after 48 hours of incubation. Bars represent standard errors of 2 repetitions.

CHAPTER 5

VARIATIONS IN UV-B TOLERANCE AMONG TEN *METARHIZIUM* SPECIES ISOLATES

Abstract

Ultraviolet radiation from the sun has the potential to reduce conidial viability of *Metarhizium* spp., a group of entomopathogenic fungi. Consideration therefore should be given to each individual isolate's susceptibility to UV radiation before field use. This study evaluates and compares the tolerance of 10 *Metarhizium* spp. isolates after exposure to an ecologically realistic dose of UV-B radiation. *M. acridum* isolate ARSEF 324, and *M. robertsii* isolates DWR 346 and DWR 203 were the most tolerant to UV-B. While *Metarhizium* isolate ARSEF 1095, and DWR 313, DWR 338, and DWR 200 were the most susceptible to UV-B radiation. The results of this study demonstrate the variations in tolerance that exists among isolates of *Metarhizium*, and suggest that laboratory UV experimentation to identify highly tolerant isolates is an important step in selecting isolates appropriate for use in the habitat of a specific pest insect.

1. Introduction

The continuation of nearly all life on earth is fueled by light from the sun. Sunlight, in general, is the total spectrum of the electromagnetic radiation given off by the sun, which includes infrared, visible, and ultraviolet (UV) wavelengths. While sunlight is essential to life on earth, some wavelengths, especially within the UV range (100-400nm) can cause deleterious effects in living organisms. The UV spectrum is

divided into three wavelength intervals: UV-C (100-280 nm), UV-B (280-320nm), and UV-A (320-400nm). Owing to absorption by the atmosphere, wavelengths within the UV-C range and a large portion of UV-B rarely reach the earth's surface.

Fractions of ultraviolet irradiation (UV-B and UV-A) from the sun that reach the earth's surface can, and often do, damage and reduce viability of *Metarhizium* spores. For example, exposure of conidia to direct sunlight at midday in midsummer caused near total inhibition of germination of most isolates examined, even after exposure of only a few hours (Braga et al., 2001b). Laboratory studies with UV-B and UV-A artificial lamps verify that the lethal elements in sunlight are primarily the UV components (Alves et al., 1996, 1998; Braga et al., 2001a, 2001c, 2002, 2006; Miller et al., 2004; Rangel et al., 2004, 2005b; Roberts and St. Leger, 2004; Ghajar et al., 2006; Fernandes et al., 2007). Of the two fractions, UV-B is considered more biologically damaging because of its direct interaction with DNA, while UV-A causes damage indirectly, primarily by inducing production of reactive oxygen species (ROS) which in turn cause oxidative damage to proteins and many other intra-cellular compounds (Griffiths et al., 1998; Braga et al., 2001b; Roberts and St. Leger, 2004). Many *Metarhizium* isolates are highly susceptible to both UV-A and UV-B, and contact with either can cause delayed germination after only 1 hour exposure to artificial UV lamps or direct sunlight, and significant reduction in viability after only 2 hours (Braga et al., 2001a, 2001b; Rangel et al., 2004, 2006, 2006a). Some isolates of *M. acridum*, like ARSEF 324, are more tolerant to UV-A and -B radiation and have greater than 50% germination after 4 hours of UV-B exposure (Braga et al., 2001c; Rangel, 2006).

Selecting isolates with increased tolerance to UV radiation in the laboratory could hasten new isolates with high field effectiveness. Due to the large variation in UV-response among isolates of *Metarhizium*, evaluating tolerance and susceptibility to ultraviolet irradiation is important to avoiding poor field efficacy. Accordingly, this study assesses the effect of UV-B radiation on ten *Metarhizium* isolates.

2. Materials and methods:

2.1. Fungal isolates:

Ten isolates of *Metarhizium* were used in this study, two of which (ARSEF 324 and ARSEF 1095) were received from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). Bischoff et al. (2009) identified ARSEF 324 as *M. acridum* and ARSEF 1095 as *M. brunneum*. The remaining eight specimens are cataloged in the Donald W. Roberts culture collection (DWR) (Utah State University, Logan, Utah). Fernandes et al. (unpublished) using the methodology of Bischoff et al. (2009), identified DWR 200, DWR 203, DWR 312, and DWR 313 as *M. guizhouense*; DWR 261 as *M. brunneum*; and DWR 338, DWR 346 and DWR 356 as *M. robertsii* (Table 2.1). Stock cultures were maintained at 4°C in test-tube slants of Potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9.

2.2 *Conidia suspensions*

Conidia of each isolate were produced on 23 ml PDAY medium in 95 x 15 mm polystyrene Petri Dishes. Isolates were grown for 14 days at 28°C prior to experiment initiation. Conidia were harvested using a microbiological loop and suspended in 0.01% Tween 80 (Sigma-Aldrich). Suspensions were vortexed and passed through an Isopore™ Membrane filter (25-mm diameter, 8-μl pore size, Millipore, Billerica, MA, USA) to remove hyphae, spore aggregates and germinated conidia. Suspensions were used immediately.

2.3 *UV-B tolerance*

For each of the three trials, 20 μl of suspension was transferred onto agar medium [4-ml PDAY with 0.002% Benomyl active ingredient (Hi-Yield Chemical Company, Bonham, TX)] in 35 x 10 mm Petri plates (Milner et al., 1991; Braga et al., 2001a, 2001b).

The plates were immediately exposed to 768mWm^{-2} of Quaité (Quaité et al., 1992a, 1992b) weighted UV-B irradiation produced by two TL 20W/12 RS fluorescent lamps (Philips, Eindhoven, Holland) [with primarily UV-B (peak at 313 nm) with minimal UV-A radiation output] in a Percival growth chamber (Boone, IA, USA) at 25 ± 1 °C. That level of irradiance affords a UV-B dosage of 2.75kJm^{-2} per hour, which approximates noon sunlight on 20 April in Logan, Utah (41.5°N latitude, 1.5 km elevation) (Braga et al., 2001c). The irradiated plates were covered with a 0.13 mm-thick cellulose diacetate film (JCS Industries, Le Mirada, CA) which blocked radiation below 290 nm. This filter permits passage of most UV-B (290–320 nm) and UV-A (320–

400 nm), but blocks UV-C (200–280 nm) and short-wavelength UV-B (280–290 nm). Spectroradiometer readings at 5-cm intervals across the irradiation target area detected a drop off of radiation intensity at the margins. Accordingly, the Petri plates with conidia were moved at fixed intervals in a predetermined pattern, so the same total irradiation (in kJ m^{-2}) was afforded for each plate. Spectral irradiance was measured with an Ocean Optics USB 2000 Spectroradiometer (Dunedin, FL).

After exposure, plates were placed at 28°C and allowed to grow for 48h. Relative germination was calculated in comparison with control (no UV) conidia. Germination (visible germ tube under 400 × magnification) was assessed for 300 conidia on each plate.

2.4 Statistical analysis

The effect of UV irradiation on spore survival was assessed using a one-way ANOVA with complete block design. The assumptions of normality and homogeneity of variance were met without transformations. Mean comparisons among treatments were adjusted for family-wise Type I error rate using the Tukey-Kramer method to maximize power to detect potential differences; *P*-values less than 0.05 were considered as significant. Data analyses were generated using the MIXED procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows.

3. Results

Tolerance to UV-B irradiation varies among isolates of the same *Metarhizium* species. After 1-hour exposure to UV-B irradiation, most isolates were only slightly affected (Figure 5.1). The most susceptible being DWR 338, DWR 200, and DWR 313

which achieved only 76.1, 72.9, and 61.9% relative germination, respectively. All other isolates had above 80% relative germination with DWR 261 achieving 99.9%. Eight of the ten isolates tested did not differ significantly after 1 hour exposure.

Two-hour exposures had a greater effect on many of the isolates (Figure 5.2). The least susceptible was ARSEF 324 with 89.2% germination; DWR 346 with 77.9% germination also demonstrated relative high tolerance. Isolates DWR 313, ARSEF 1095, DWR 338 and DWR 200 were the most susceptible, realizing 28.3, 27.8, 27.1, and 10.6% relative germination, respectively ($P < 0.0001$). DWR 203, DWR 312, DWR 261, and DWR 356 managed 62.9, 60.3, 49.3, and 47.6% germination respectively. Four levels of statistical significance were evident among the isolates but the isolate groups overlapped somewhat. ARSEF 324, DWR 346, and DWR 203 formed the most tolerant group. The second group was comprised of DWR 346, DWR 203, DWR 312, DWR 261, and DWR 356. The third group consisted of DWR 261, DWR 356, DWR 313 ARSEF 1095, and DWR 338; while the last (least UV-B tolerant) group was DWR 313, ARSEF 1095, DWR 338, and DWR 200.

4. Discussion

After one-hour exposure, very few differences were seen among these 10 isolates; therefore this duration of exposure is insufficient to separate UV-tolerant isolates from intolerant ones. However, the variation seen after two-hour exposures clearly revealed the range of UV tolerance present in otherwise similar isolates of *Metarhizium*. Isolates DWR 200 and DWR 203, which have been shown to be closely related genetically (Fernandes et al., 2010), have significantly different responses to UV radiation. The

sensitivity to UV-B radiation of many *Metarhizium* isolates potentially could be extremely harmful to biological control efforts based on such isolates.

Several factors may play key roles in increasing an isolate's tolerance to UV-B exposure. Braga et al. (2006) noted that wild type green conidia of ARSEF 23 were more tolerant to UV radiation than mutants that had changed or lost their color pigmentation; but the green pigment of a closely related isolate (ARSEF 2575) apparently provides little UV-B protection for its conidia (Rangel et al., 2006b). Rangel et al. (2004; 2006a) demonstrated that UV tolerance was increased by twofold after producing *Metarhizium* conidia spores on nutrient deprived media, and that conidia obtained from an insect cadaver was more susceptible to UV than *Metarhizium* conidia produced on nutrient-rich artificial media. Understanding these attributes will help in selecting the most appropriate fungal isolate and conidial mass-production method, to develop biological control products with optimized field UV tolerance. In addition, formulation components, when properly selected and used, are known to enhance UV protection. For example, Inglis et al. (1995) noted that *Beauveria* spores formulated with fluorescent brightener, Tinopal LPW and clay emulsion had an increased survival after UV exposure.

Several studies report that *M. acridum* isolates, particularly ARSEF 324, are more tolerant to UV radiation than other species of *Metarhizium*. For example, after 4h of UV-B exposure ARSEF 324 exhibited 20% greater relative culturability than any of 29 non-*acridum* *Metarhizium* isolates (Braga et al., 2001c); and ARSEF 324 was consistently highly tolerant to the same UV-B dose regardless of the growth media used for conidia production (Rangel et al., 2005a). Miller et al. (2004) suggested that the high tolerance

of ARSEF 324 is due perhaps to different isozyme profiles for catalase-peroxidase, glutathione reductase and superoxide dismutase, which were reduced less than in non-UV tolerant strains. ARSEF 324 has likely undergone natural selection to survive the harsh environmental conditions of Australian locust habitats, making it an ideal candidate for use against locust and other orthopteran pests worldwide.

An important factor that was not tested in this study is the possibility of post-stress growth delay resulting from sub-lethal UV treatments. Nevertheless, several other authors have observed this phenomenon and commented the potential harm it could cause (Zimmermann, 1982; Moore et al., 1993; Alves et al., 1998). Fernandes et al. (2007) deduced that this observed delay indicated that surviving conidia required a period of time to recover from the UV stress before resuming normal growth. Braga et al. (2001c) suggested that this delay would likely reduce virulence and increases the chance of successful host defenses. Braga et al. (2001a) further postulated that this delay may likely be a result of an induced set of responses to facilitate DNA repair. Whatever the molecular basis for this delay, the expected downstream results are clear; infection and disease development surely also would be delayed at the minimum.

The present study clearly identifies that variation in UV-radiation tolerance exists between species and even within a species of *Metarhizium*. Of the isolates tested in this study, ARSEF 324, DWR 346 and DWR 203 were the most tolerant to UV and, therefore, in field conditions would be less affected by UV radiation from the sun than the other seven isolates, and more likely to achieve insect infection than isolates which were highly impeded by UV exposure. The methods used in this study are relatively

simple and inexpensive, but provide clear and distinct results. Traditional selection techniques such as virulence and culturability would give no indication of an isolate's susceptibility to UV-radiation, and thus potentially ignore a critical pitfall to successful field use. Evaluation of an isolate's tolerance to UV radiation therefore is an obvious step in the selection process.

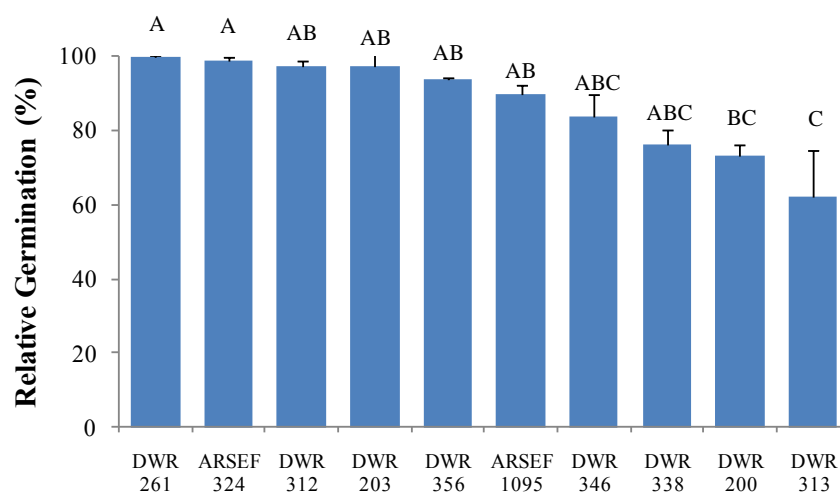


Figure 5.1 Relative percent germination of conidia after one hour exposure to UV-B radiation (total dose = 2.8 kJm^{-2}) of ten *Metarhizium* spp. isolates. Data represent the average of 3 trials done on separate days with different cultures of each isolate. Variation among replicates is indicated by standard error bars. Statistically significant differences are presented by letter designations below the x-axis.

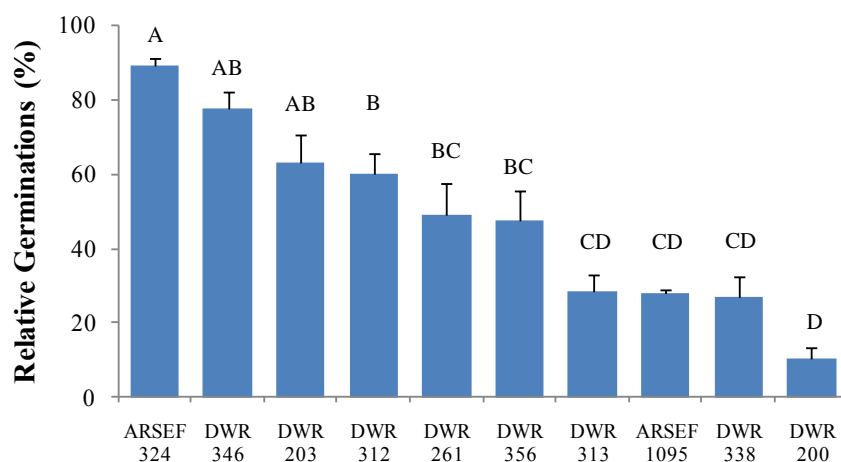


Figure 5.2. Relative percent germination of conidia after two hours exposure to UV-B radiation (total dose = 5.5 kJm^{-2}) of ten *Metarhizium* spp. isolates. Data represent the average of 3 trials done on separate days with different cultures of each isolate. Variation among replicates is indicated by standard error bars. Statistically significant differences are presented by letter designations below the x-axis.

CHAPTER 6

SPEED OF CONIDIAL GERMINATION OF TEN *METARHIZIUM* SPECIES ISOLATES AT HIGH AND LOW TEMPERATURES

Abstract

Fungal germination is the process in which growing hyphae (germ tubes) emerge from previously dormant spores. For *Metarhizium*, aerially produced conidia are the growth stage preferred for wide-scale field application; and conidial germination is the first step that must occur in order for *Metarhizium*-mediated biological control programs to be successful. This study evaluates the speed of germination for eight new *Metarhizium* spp. isolates from the United States, plus two commercialized isolates (ARSEF 324 and ARSEF 1095). Germination was assessed at 16°, 28°, 32°, 36°C, and GT_{50s} (time to 50% germination) were calculated for each isolate. DWR 203, ARSEF 1095, DWR 261, DWR 346, and DWR 356 were the fastest germinating isolates in this study. ARSEF 324, while not the fastest germinator, germinated at 36° nearly as fast as it did at 32°, and faster than it did at 28°C. This high heat tolerance will be important to field use of this isolate in exceptionally hot climates. Evaluations of several traits, e.g. heat and UV tolerance, are necessary to predict overall potential; but germination speed also is important, and those isolates with the fastest germination rates probably would be the better candidates as biological control agents in the field.

1. Introduction

Entomopathogenic fungi have demonstrated great potential in laboratory settings as control agents for pest insects. Unfortunately, field trials too often yield disparaging results largely due to fluctuating environmental conditions which hamper pathogen effectiveness. Ambient temperature may be a key infection-impediment for entomopathogenic fungi due to its effect on, among other processes, metabolic activity supporting spore germination. Temperature has long been seen as a factor determining the level and rate of conidial germination of entomopathogenic fungi (Luz and Fargues, 1997; Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Yeo et al., 2003; Bugeme et al., 2008; Leemon and Jonsson, 2008). When selecting a particular fungal isolate for use as a biocontrol agent, the temperature range at which it can grow should be a primary consideration.

The term germination, as applied to fungi, refers to the “processes and changes occurring during the resumption of development of a resting structure and its transformation to a morphologically different structure” (Allen, 1965). Generally, germination has occurred if a newly developing hypha, known as a germ tube, can be seen emerging from the spore. While relatively few studies have treated the metabolic aspect of germination in fungi, understanding the mechanism behind this phenomena will aid in the more effective use of entomopathogenic fungi for pest control. Paul Allen (1965) distinguished three stages of fungal germination: (1) a preliminary stage of swelling, (2) the emergence of the germ tube, and (3) the early development of the germ tube after its emergence. He also noted that temperature often plays an integral part in

the activation of the spore from its dormant state. Furthermore, the second phase of spore swelling has been shown to be temperature-sensitive (Yanagita, 1957).

Rapid germination and hyphal growth rates are believed to be advantageous to fungal biological control agents because these traits are expected to encourage rapid host infection (Hajek and St. Leger, 1994; Varela and Morales, 1996; Liu et al., 2003). New isolates are often subjected to laboratory tests to evaluate both their optimal temperatures for germination and the effect of extreme temperatures (both high and low) on germination. The optimal temperature for *Metarhizium* spp. germination is usually between 25° and 30°C (Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Yeo et al., 2003; Bugeme et al., 2008). The upper temperature limit for conidial germination of *Metarhizium* isolates is from 35 to 37°C (Fargues et al., 1997; Luz and Fargues, 1997; Milner, 1997; Ouedraogo et al., 1997; Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Milner et al., 2003). The lower temperature threshold for *Metarhizium* spp. ranges 8 to 10°C (De Croos and Bidochka, 1999; Tefera and Pringle, 2003; Yeo et al., 2003; Fernandes et al., 2008, 2010). These studies noted that while extreme cold and hot temperatures do permit growth, germination was significantly reduced and/ or delayed.

The first step in host infection i.e., spore transformation from dormancy to activity, is temperature dependent. Isolate selection based on germination rates and range that match a target pest's habitat will greatly augment the potential for successful control. The current study evaluates and compares ten *Metarhizium* spp. isolates for speed of germination at 16, 28, 32, and 36°C.

2. Materials and methods

2.1. Fungal isolates

Ten isolates of *Metarhizium* were used in this study, two of which (ARSEF 324 and ARSEF 1095) were received from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). Bischoff et al. (2009) identified ARSEF 324 as *M. acridum* and ARSEF 1095 as *M. brunneum*. The remaining eight specimens are cataloged in the Donald W. Roberts culture collection (DWR) (Utah State University, Logan, Utah). Fernandes et al. (unpublished) using the methodology of Bischoff et al. (2009), identified DWR 200, DWR 203, DWR 312, and DWR 313 as *M. guizhouense*; DWR 261 as *M. brunneum*; and DWR 338, DWR 346 and DWR 356 as *M. robertsii* (Table 2.1). Stock cultures were maintained at 4°C in test-tube slants of Potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9.

2.2 Conidial suspensions

Conidia of each isolate were produced on 23 ml PDAY medium in 95 x 15 mm polystyrene Petri dishes. Isolates were allowed to grow for 14 days at 28°C prior to experiment initiation. Conidia were harvested using a microbiological loop and suspended in 0.01% Tween 80 (Sigma-aldrich). Suspensions were vortexed and passed through Isopore™ Membrane filters (25-mm diameter, 8-µl pore size, Millipore, Billerica, MA, USA) to remove hyphae, spore aggregates and germinated conidia. Suspensions were used immediately for germination studies.

2.3 Germination

For each of the three trials, 20 μ l of the conidial suspension was transferred onto media (4 ml) PDAY with 0.002% benomyl (active ingredient) (Hi-Yield Chemical Company, Bonham, TX) (Milner et al., 1991; Braga et al., 2001a, 2001b) in 35 x 10 mm Petri plates. For each isolate, individual plates were prepared for each combination of time and temperature treatment. Four temperatures were tested $16^{\circ}\text{C} \pm 1$, $28^{\circ}\text{C} \pm 1$, $32^{\circ}\text{C} \pm 1$, and $36^{\circ}\text{C} \pm 1$. Every 4 hours for 28 hours for each isolate at each temperature a plates were removed and percent germination was determined by adding a few drops of methyl blue in lactophenol to the agar surface to stop (kill) the spore growth, and covering the inoculated area with a glass cover slip to facilitate observed germination at $400\times$ magnification. Percent germination was determined by observing 300 spores per plate. Emergence of a germ tube through the spore wall was scored as germination of that spore.

2.4 Statistical analysis

Time to 50% germination (GT_{50}) was calculated separately for each replication of each combination of temperature and isolate. The data for each replicate trial was truncated prior to calculation, viz. consecutive time values with repeated percent germination scores, usually either 0% or 100%, were removed (i.e., if 100% germination was reached at hour 16, the following time measurements, which were also 100%, were not included in the analysis). The effects of temperature on GT_{50} s of the different isolates were assessed using an analysis of variance of a two-way factorial in a split-plot design with whole plots in blocks. The whole plot unit was an incubator, and the whole plot

factor was temperature. Four incubators, each set at a different temperature, were blocked by replication. Plates within the incubators were the subplot units, and isolate was the subplot factor. Assumptions of normality and homogeneity of variance were evaluated using residual analysis. Due to apparent heteroscedasticity, heterogeneous variances were estimated for levels of temperature by partitioning the whole plot variance. Assumptions of normality appeared adequately met. Mean comparisons were unadjusted for family-wise Type I error rate to maximize power to detect potential differences; *P*-values less than 0.05 were considered as significant. Data analyses were generated using the PROBIT and MIXED procedures in SAS/STAT® software, Version 9.1.3 of the SAS System for Windows.

3. Results

3.1 Germination at 16°C

At 16°C two isolates (ARSEF 1095 and DWR 261) had greater than 80% germination after 16 hours. Both of these isolates reached >95% after 20 hours incubation. Isolates DWR 203, DWR 261 DWR 346, DWR 356 all reached >95% germination after 28 hours at 16°C. *M. acridum* isolate ARSEF 324 achieved only 58% germination by hour 28, while DWR 312, DWR 338, DWR 200 and DWR 313 had after 28 hours germinated 67, 46, 25, and 14%, respectively (Figure 6.1).

Estimates for the amount of time it would require an isolate to achieve 50% germination (GT₅₀) varied among isolates. At 16°C, ARSEF 1095, DWR 261, DWR 346, DWR 203, and DWR 356 were the fastest and were not significantly different from each other (Figure 6.2a). DWR 312, ARSEF 324, and DWR 338, were the next group,

requiring greater than 25 hours to attain 50% germination. The slowest isolates to germinate at this temperature were DWR 200, and DWR 313, taking 33.19 and 34.62 hours to reach 50% germination, respectively (Table 6.1).

3.2 Germination at 28° and 32°C

There was no significant difference between 28 and 32°C. All of the isolates preferred this temperature range for growth, except *M. acridum* isolate ARSEF 324 which grew the fastest at 36°C. After 12 hours, DWR 203 and ARSEF 1095 reached >95% germination. By hour 16, DWR 261, 346, and 356 achieved >95% germination, while DWR 312 took 24 hours to reach that level. After 28 hours at 28°C, isolates DWR 338, ARSEF 324, DWR 200 and DWR 313, achieved 90, 68, 66, and 47% germination, respectively. While after the same amount of time at 32°C, these isolates germinated 80, 75, 74, and 65%, respectively.

At both 28° and 32°C, the isolate rankings were the same, with DWR 203 being the fastest and DWR 313 the slowest (Figure 6.2b & c). The most clearly different (statistically significant) levels of GT_{50} 's were seen at 28°C. The five fastest germination isolates were not significantly different, ranging in time to 50% germinating from 8.89 to 10.41 hours, and included DWR 203, ARSEF 1095, DWR 261, DWR 346, and DWR 356. The remaining five isolates were all statistically independent from each other and ranged from 15.10 (DWR 312) to 29.02 (DWR 313) hours.

3.3 Germination at 36°C

After incubating only 16 hours at 36°C, isolates DWR 203 and DWR 356 attained >95% germination. After 24 hours, DWR 261, DWR 346 and DWR 312 realized >90% germination. ARSEF 324 reached 74% germination at 28 hours, while DWR 338, DWR 200, ARSEF 1095, and DWR 313 scored 74, 52, 51, and 40% germination (Figure 6.1). GT_{50} for isolates at 36°C ranged from 9.27 hours (DWR 203) to 30.82 hours (DWR 313) (Figure 6.2d).

4. Discussion

This study demonstrates that variability exists among isolates of *Metarhizium* in regards to speed of germination and ability to germinate at different temperatures. *Metarhizium* is most environmentally persistent as conidia, and thus conidiospores are the stage generally produced for field (Auld, 1992; Jenkins and Goettel, 1997; Lomer et al., 2001; Roberts and St. Leger, 2004). Spore germination, therefore, is the primary step in disease initiation; and selecting isolates with the ability to germinate efficiently (quickly and to high levels at a wide range of temperatures) is crucial in attaining successful insect control.

Past studies have indicated that the optimal temperature for germination of *Metarhizium* is usually between 25° and 30°C (Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Bugeme et al., 2008). This study observed that nearly all of the isolates tested germinated fastest at 32°C (DWR 356 was slightly faster at 28°C), but GT_{50} did not differ greatly between 28° and 32°C (Table 6.1). This range of temperatures clearly is optimal for germination of the isolates examined here.

The isolates that were able to germinate the fastest at optimal temperatures were DWR 203, ARSEF 1095, and DWR 261. Fast germination rates have been related in other hyphomycete fungi to virulence (Al-Aidroos and Roberts, 1978; Samuels et al., 1989). Germination, however rates will differ between nutrient-rich media and the nutrient-poor insect cuticle. Rangel et al. (2008a) observed that conidia actually germinated faster on nutrient-deprived media compared to nutrient-rich media. Yeo et al. (2003) pointed out that germination is not exclusively dependent on temperature, but also on the presence of water and is likely influenced by cuticular components, making it difficult to directly relate in-vitro findings with *in-vivo* activities of pathogens.

Perhaps the most useful aspects of *in vitro* germination studies are to delineate the temperature extremes at which isolates can function. While at 16°C all the isolates germinated much slower than at their optimal temperatures, their rankings relative to each other were the same, i.e., the fastest isolates at 28°C were also the fastest isolates at 16°C. Perhaps adding a lower temperature to the study would have been useful in better identifying if any of the isolates possess an exceptional ability to germinate at extreme low temperatures. Many of the isolates germinated well at 36°, which is near the upper threshold for *Metarhizium* spp. growth (Fargues et al., 1997; Luz and Fargues, 1997; Milner, 1997; Ouedraogo et al., 1997; Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Milner et al., 2003). Three isolates, ARSEF 324, DWR 312, and DWR 356, had nearly the same GT₅₀ at 36° as at 32°, indicating that germination of these isolates was not affected greatly by the increase in temperature. Interestingly, ARSEF 1095, one of the faster germinators at optimal and low temperatures was significantly

slower at 36°C. This was the only significant ranking change observed among these ten isolates at the four temperatures.

M. acridum (isolate ARSEF 324) did not exceed 80% germination at any temperature, indicating a low viability or slow germination in the conidia used. Rangel et al. (2005b) observed that ARSEF 324 was able to reach > 90% germination within 12 hours at 28, 32, and 35°C, using the same nutrient source as this study. The batch of ARSEF 324 conidia used in the current study had reduced vigor in comparison to those used by Rangel et al., but the studies are comparable in that conidia germinated almost as fast at 36° as it did at 32°C. *M. acridum* isolates have been extolled for their ability to withstand and grow at high temperatures, and this trait is one of the major reasons that they are used for control of orthopteran pest insects that live in high-temperature regions (Milner, 1997).

Ambient temperature is a driving force in the germination of entomopathogenic fungi. As observed in this study, isolates vary drastically in relation to germination speed and ability to germinate at different temperatures. Of the *Metarhizium* isolates used in this study DWR 203, DWR 261, DWR 346, DWR 356 and ARSEF 1095 germinated quickest; and, with the exception of ARSEF 1095, were consistently fast at the various temperatures. While more evaluations of isolate characteristics are necessary to predict overall potential, based on germination, these isolates would be good candidates for development as biological control agents in the field.

Table 6.1 The average time required to achieve 50% germination (GT₅₀) for ten *Metarhizium* spp. isolates at four temperatures, calculated separately for each isolate at each temperature. Temperatures include (a) 10°C, (b) 28°C, (c) 32°C and (d) 36°C. Data analyses were generated using the PROBIT procedures in SAS/STAT® software, Version 9.1.3 of the SAS System for Windows.

	DWR 200	DWR 203	DWR 261	DWR 312	DWR 313	DWR 338	DWR 346	DWR 356	ARSEF 324	ARSEF 1095
16°	33.19	15.38	12.58	25.77	34.62	28.42	14.99	15.56	26.91	12.33
28°	23.57	8.89	9.48	15.10	29.02	21.53	10.18	10.41	18.00	9.14
32°	21.48	8.29	8.99	13.03	23.28	20.31	9.84	10.55	16.32	8.70
36°	25.26	9.27	10.74	13.38	30.82	22.82	11.64	10.84	16.36	22.50

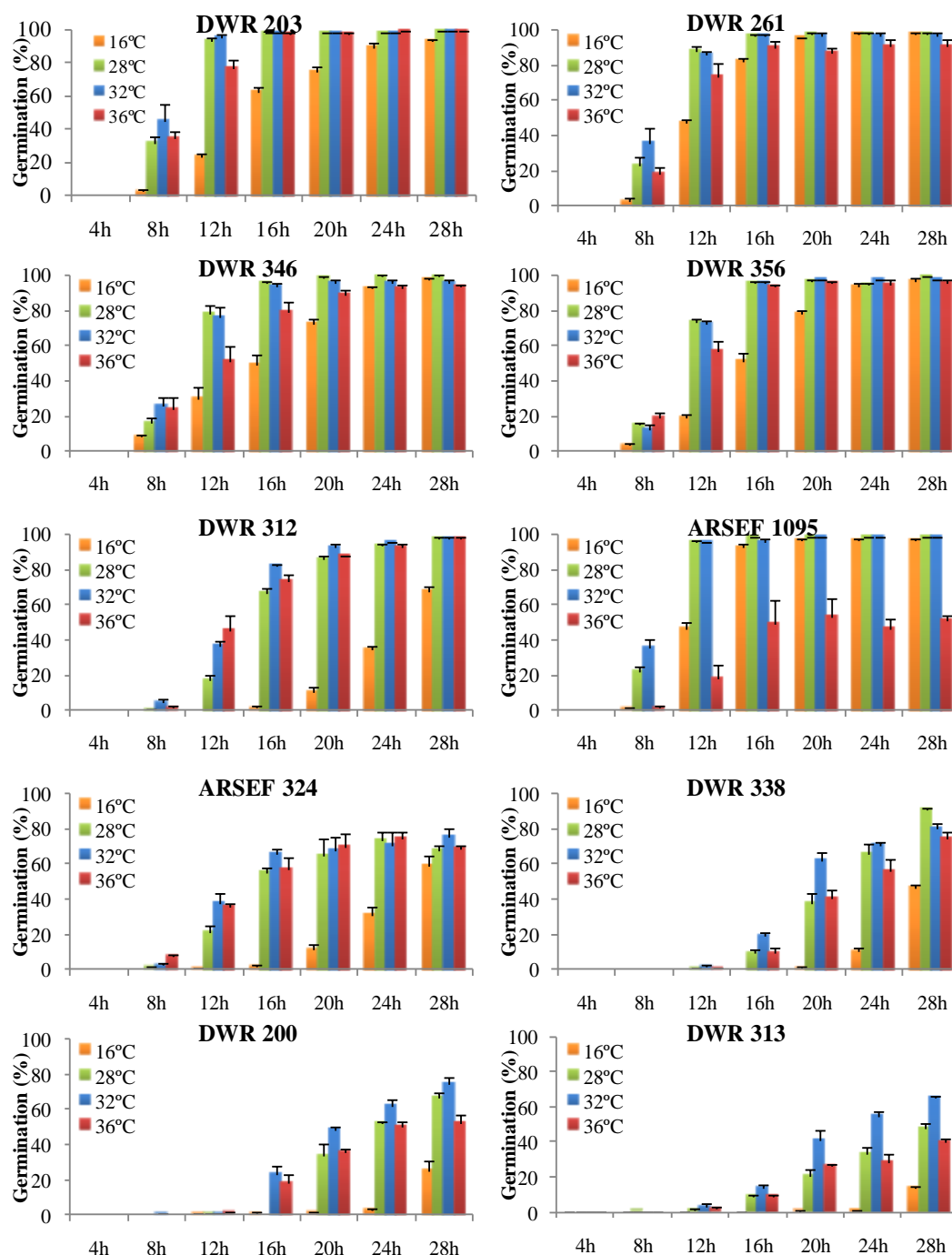


Figure 6.1 The average percentage of conidia germinated at four hour increments between 4 and 28 hours incubation time for ten *Metarhizium* spp. isolates. Germination was observed at 4 temperatures: 16°, 28°, 32°, and 36°C. Variation among three replicates is indicated by standard error bars.

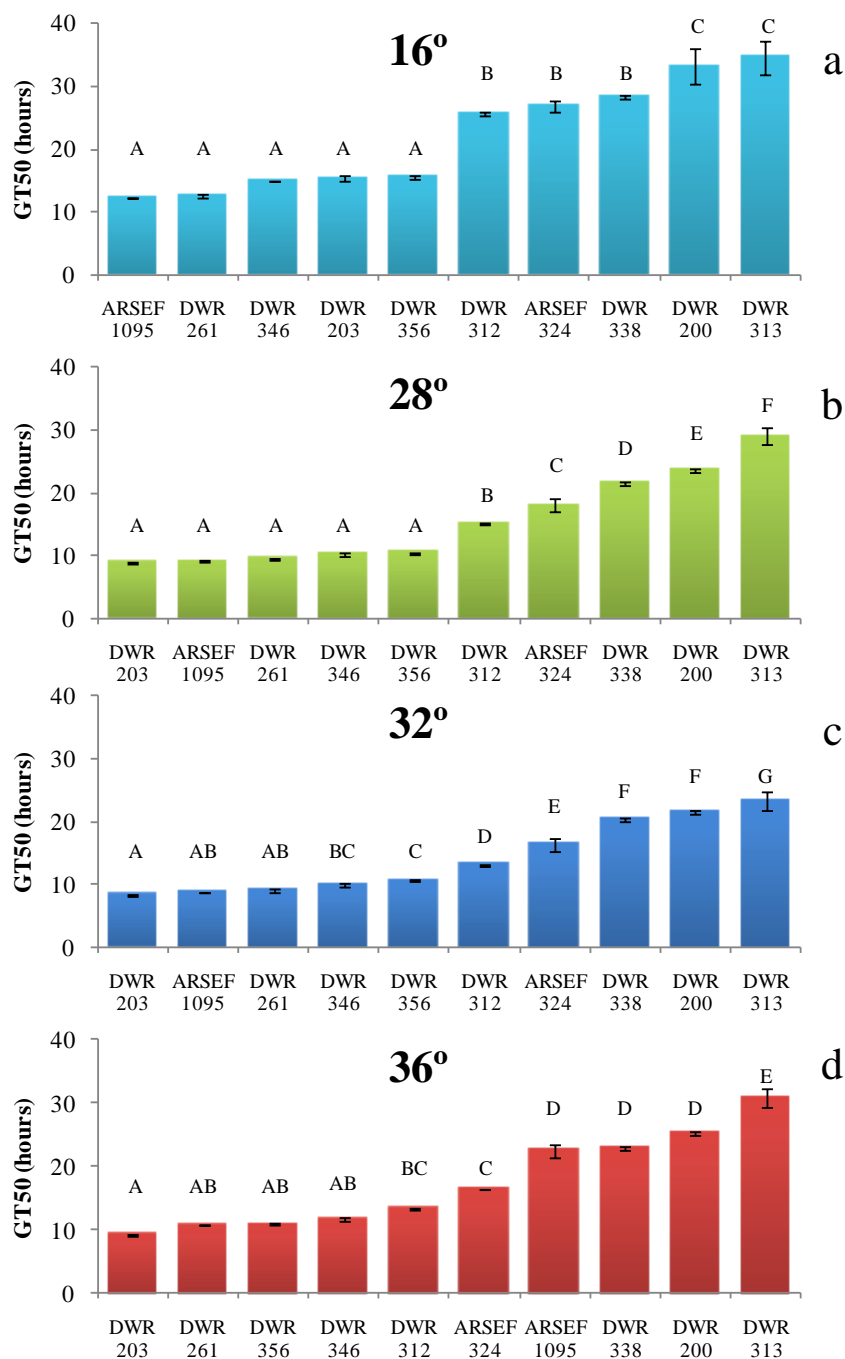


Figure 6.2 The average time required to achieve 50% germination (GT₅₀) for ten *Metarhizium* spp. isolates at four temperatures, calculated separately for each isolate at each temperature. Temperatures included (a) 10°C, (b) 28°C, (c) 32°C and (d) 36°C. Statistically significant differences are presented by letter designations below the x-axis.

CHAPTER 7

EFFECT OF TEMPERATURE ON THE GROWTH RATES OF TEN *METARHIZIUM* SPECIES ISOLATES

Abstract

Among the key factors that should be investigated when devising a new microbial control program is the temperature tolerances, high and low, of the candidate fungi; since the isolate(s) selected must have rapid rates of growth at various temperatures encountered in the habitat of target insect hosts. This study evaluates ten *Metarhizium* spp. isolates (eight new isolates from USA soil; and two commercial isolates, ARSEF 324, and ARSEF 1095) for mycelial growth rates at six temperatures (20°, 24°, 28°, 32°, 36°, and 40°C). Arizona isolate DWR 203 had the greatest overall growth rate (mm/day) at most temperatures, while ARSEF 324 grew better than all others at 36°C (1.6 mm/day). None of the tested isolates were able to grow at 40°C. ARSEF 1095 grew well at 20, 24, and 28°C, but grew poorly at 32° or higher. Growth rates at different temperatures are good indicators of how quickly and effectively an isolate can cause infection in an insect. By harmonizing pathogen growth rates with likely pest-ambient temperature, the potential for successful fungal mediated biological control will be enhanced.

1. Introduction

Fungal pathogens infecting their insect hosts must adapt to several different environments throughout the infection process, and they have evolved the ability to respond to these changing micro-environments through different biochemical processes

and cellular differentiation that result in specific morphological structures. Conidia, upon finding a suitable host will: (a) attach to the cuticle; (b) germinate to produce a germ tube; (c) form an appressoria, which utilizes cuticle degrading enzymes and mechanical pressure to penetrate the insect epicuticle; and finally, (d) colonize the host through vegetative hyphal elongation and hemocoel-mediated dispersal of blastospores (Hajek and St. Leger, 1994). As with germination, the rate at which an isolate can complete each stage of host-infection will greatly determine its efficacy in pest control. Accordingly, new isolates are often evaluated based on rate of vegetative (hyphal) growth.

Hyphal elongation occurs at the hyphal apex (tip). The process involves weakening of the existing wall via enzymic degradation, materials being delivered in vesicles or vacuoles to the tip and inserted into the existing wall, and then tip extension due to osmotic pressure (Weber and Hess, 1976; Cole and Hoch, 1991). Like most metabolic activities, the rate of vegetative growth in insect pathogenic fungi is influenced by ambient temperatures (Ouedraogo et al., 1997; Ekesi et al., 1999; Hallsworth and Magan, 1999; Smits et al., 2003; Brooks et al., 2004).

Insects are found in nearly all habitable climates on earth (Pedigo, 2002). When selecting a fungal biological control agent for field use against a pest insect the thermal growth preferences of that isolate should be in tune with the thermal conditions of the target insect (Ouedraogo et al., 1997; Roberts and St. Leger, 2004). Consequently, vegetative growth studies are needed to examine an isolate's (a) optimal growth temperature; (b) rate of growth at various temperatures; and (c) the effects of extreme temperatures on growth rate. While significant variation exists between strains of

Metarhizium spp. with regard to vegetative growth rates, several previous studies have determined that the optimal temperature for germination and vegetative growth are often closely related, and usually range between 25° and 30°C for this fungal genus (Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Yeo et al., 2003; Bugeme et al., 2008). The current study evaluates and compares the rate of vegetative growth at six temperatures (20, 24, 28, 32, 36, and 40°C) for ten *Metarhizium* spp. isolates.

2. Materials and methods

2.1 Fungal isolates

Ten isolates of *Metarhizium* were used in this study, two of which (ARSEF 324 and ARSEF 1095) were received from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). Bischoff et al. (2009) identified ARSEF 324 as *M. acridum* and ARSEF 1095 as *M. brunneum*. The remaining eight specimens are cataloged in the Donald W. Roberts culture collection (DWR) (Utah State University, Logan, Utah). Fernandes et al. (unpublished) using the methodology of Bischoff et al. (2009), identified DWR 200, DWR 203, DWR 312, and DWR 313 as *M. guizhouense*; DWR 261 as *M. brunneum*; and DWR 338, DWR 346 and DWR 356 as *M. robertsii* (Table 2.1). Stock cultures were maintained at 4°C in test-tube slants of Potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9.

2.2 Conidial suspension:

Conidia of each isolate were produced on 23 ml PDAY medium in 95 x 15 mm polystyrene Petri dishes. Isolates were allowed to grow for 14 days at 28°C prior to experiment initiation. Conidia were harvested using a microbiological loop and suspended in 0.01% Tween 80. Suspensions were used immediately.

2.3 Vegetative growth

For each of three trials, 1 µl of suspension was placed in the center of a Petri dish (23 ml PDAY 95 x 15 mm polystyrene Petri plates). For 24 hours, conidia were allowed to germinate at 28°C, and then moved to their respective temperatures. Temperature treatments included 20°C ±1, 24°C ±1, 28°C ±1, 32°C ±1, 36°C ±1, and 40°C ±1. Colony growth was determined by measuring (mm) across two diameters for each colony every 24 hours using a carbon fiber composites digital caliper with 0.1mm resolution (±0.2, Fisher Scientific). The initial measurement was made before the first treatment and that number was subtracted from subsequent measurements.

2.4 Statistical analyses

The rate of growth of each isolate was assessed using a one-way ANOVA with complete block design for each temperature. The assumptions of normality and homogeneity of variance were met without transformations. Mean comparisons among treatments were unadjusted for family-wise Type I error using the Tukey-Kramer method to maximize power to detect potential differences; *P*-values less than 0.05 were

considered as significant. Data analyses were generated using the MIXED procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows.

3. Results

The rates of growth of the ten isolates varied when compared at the same temperature. Also, variations were noted with single isolates at different temperatures (Figure 7.1). At 20°C, growth rates (mm/day) were statistically similar among all isolates (Figure 7.2). Alaskan isolate DWR 261 had the highest rate of growth at this temperature (4.27 mm/d), but was statistically different from only two other isolates (DWR 312, and ARSEF 324). ARSEF 324 exhibited the slowest rate of growth among the test isolates at 20°C (2.73 mm/d), and differed statistically from DWR 261, 203, 356, and 346.

At 24°C there was slightly more variation among the isolates than at 20°C. Arizona isolate DWR 203, which had the fastest rate of growth at this temperature, was statistically different from all isolates except DWR 356 and DWR 346 (Figure 7.2). There was negligible change in the rates of growth at 20 and 24°C for isolates; DWR 261, DWR 338, and DWR 312 (Table 7.1). The slowest rate of growth at 24°C was a group comprised of ARSEF 324, DWR 338, and DWR 312.

The variation among isolates continued to increase at 28°C, with all the isolates increasing their rate of growth except for DWR 261, ARSEF 1095 and DWR 312. DWR 203 achieved the highest rate of growth observed (6.42 mm/day) among all temperatures and isolates tested; this statistically differed from all isolates except DWR 356 and DWR 346 (Figure 7.2). DWR 312 was slightly slower than its rate at 24°C, and again had the

lowest growth rate at this temperature (3.0 mm/day), and was significantly different from all isolates except ARSEF 324 and DWR 338.

The most statistically clear differences seen at 32°C. Four separate groups were detected: (a) DWR 203, (b) DWR 346 and DWR 356, (c) ARSEF 324, DWR 312, DWR 313, DWR 338, and DWR 200, (d) DWR 261 and ARSEF 1095 (Figure 7.3). All isolates decreased in rate of growth between 28 and 32°C except for DWR 312 and ARSEF 324. Group (d) decreased to less than half of their previous rate of growth (Table 7.1).

All rates of growth were drastically reduced at 36°C; although some isolates were still able to produce a minor amount of hyphal extension. ARSEF 324, DWR 203, and DWR 313 had the highest rates of growth and were statically similar (Figure. 7.3). Many isolate's daily growth was so minimal it required several days before visible change could be measured; e.g., DWR 261 and ARSEF 1095 only reached a total growth of 1.71 and 2.35 mm after 10 days exposure, respectively. ARSEF 324 colonies reached almost 16 mm diameter after 10 days at 36°C, and this was the greatest growth of any isolate at this temperature.

The highest temperature treatment, 40 degrees centigrade, was too warm to allow growth. All isolates at this temperature measured at or near zero mm per day growth rate.

The growth rate for each isolate at each temperature was further calculated as a percent of the optimal temperature (highest growth rate observed for that isolate at any temperature) (Figure 7.4). Isolates with preferred growth temperature of 24°C included DWR 261 and ARSEF 1095; isolates that favored 28°C were DWR 203, DWR 346, DWR

356, and DWR 200; while those that performed best at 32°C were of ARSEF 324 and DWR 312 (Table 7.2).

4. Discussion

Temperate regions have fluctuating climates that require a pathogen to survive and infect at a wide range of ambient temperatures. Evaluating an isolate's growth response to different temperatures explicates the growth competence and a limitation of that individual isolate, and allows comparisons with other fungal strains with regard to growth rate.

One way to express the effect of temperature on an isolate's individual growth rate is as a percent of its optimal potential (Table 7.2). ARSEF 324 and DWR 338 were the two isolates which maintained growth rates most similar to that seen at their optimal (Figure 7.4). ARSEF 1095 and DWR 261 had the greatest rate changes at temperature different from their optimal. ARSEF 1095 and DWR 261 also were the only two isolates to grow fastest at 24°C. Six of the ten isolates grew best at 28°C, which agrees with several other studies stating that *Metarhizium* generally prefers temperatures between 25° to 30°C (Thomas and Jenkins, 1997; De Croos and Bidochka, 1999; Yeo et al., 2003; Bugeme et al., 2008). Only two isolates had an optimal temperature above 30°C, *M. acridum* isolate ARSEF 324 from Australia, and *M. robertsii* DWR 312 from Utah, USA. Leemen and Jonsson (2008) suggested that an above 30°C optimal temperature is critical for the success of a biopesticide. The disadvantage of only considering optimal growth temperatures is that they give no indication of effects on vegetative proliferation rates at other temperatures that might be encountered in the field. DWR 203 favored 28°C to

32°C, and grew significantly faster than any other isolate at 32°C. The two isolates that grew best above 30°C (ARSEF 324 and DWR 312) consistently had relatively slow growth rates at all temperatures (Figure 7.1).

Comparing rates of vegetative growth among isolates may help in selecting an isolate suitable for field use. At the temperature extremities of this study (20° and 40°C) there was little variation among the isolates and it is difficult to differentiate between them, while at the medium temperatures the growth rates for individual isolates becomes more distinct. DWR 203 clearly grew the fastest when given the opportunity, followed by DWR 346 and DWR 356. While ARSEF 1095 and DWR 261 grew moderately well at lower temperatures (20° to 28°C), they were severely debilitated at 32°C. Other isolates, like ARSEF 324 and DWR 338 had fairly similar rates of growth between 20° and 32°C. While slower growing than the other isolates, they were able to maintain, at non-optimal temperatures, rates similar to their optimal temperature rate.

Growth rates of all the isolates used in this study were sharply reduced by 36°C and completely stopped at 40°C. The growth deficiency observed at 40°C substantiates observations noted in several other studies (Ouedraogo et al., 1997; Hallsworth and Magan, 1999; Smits et al., 2003; Brooks et al., 2004). The *M. acridum* isolate (ARSEF 324) grew better than all isolates at 36°C with a rate of 1.6 mm/d. Ouedraogo et al. (1997) also found that ARSEF 324 had minimal yet measurable growth at 35°C, and observed that in general, *M. acridum* (= *M. flavoviride*) isolates were more heat tolerant than *M. anisopliae* s.l. In many areas where a biological control agent might be used, 36°C is likely to be a common temperature, especially among orthopteran pests, which

are known to use sunlight to raise their body temperatures to ward off infection (Parker, 1982; Kemp, 1986; Whitman, 1987, 1988; Prange and Pinshow, 1994; Thomas and Jenkins, 1997; Arthurs and Thomas, 2001; Blanford and Thomas, 2001; Ouedraogo et al., 2002, 2004; O'Neill and Rolston, 2007). DWR 203, DWR 313, DWR 200, and DWR 346 also had some growth at 36°C, and may be candidates for field use based on that criterion. ARSEF 1095, a commercialized USA isolate, did not perform well at higher temperatures ($\geq 32^\circ$), this could potentially cause problems in many field situations where if the temperature is expected to be above this threshold.

Of the isolates in this study, DWR 203 had the highest rate of growth and grew well between 20-32°C. ARSEF 324 grew the best at high temperatures and also maintained a similar rate of growth between 20-32°C. Inglis et al. (1996) found a strong correlation between vegetative growth of *Beauveria bassiana* and mycosis of grasshoppers. However, Ouedraogo et al. (1997) indicated in further studies that hyphal growth was a relatively poor predictor of mortality with *M. acridum* isolates. While effects of temperature on growth rates are likely a factor in infection effectiveness, host-pathogen interactions (e.g., behavioral fever) which likely will also play a role in infection success should be considered.

The data reported here will be most useful for comparison with temperature data relating to the target pest and the proposed treatment area. By harmonizing pathogen growth rates with likely ambient temperatures, the predicting of potential for successful biological control with a particular isolate will be significantly improved.

Table 7.1 The growth rate (mm/day) of ten *Metarhizium* spp. isolates at six different temperatures.

	DWR 200	DWR 203	DWR 261	DWR 312	DWR 313	DWR 338	DWR 346	DWR 356	ARSEF 324	ARSEF 1095
20°	3.22	4.17	4.27	3.01	3.10	3.18	3.91	4.12	2.73	3.51
24°	4.23	5.66	4.62	3.16	4.34	3.44	5.20	5.24	3.49	4.47
28°	4.58	6.42	4.14	3.00	5.60	3.54	5.59	5.76	3.88	4.28
32°	3.30	5.68	1.43	3.69	3.69	3.42	4.87	4.77	4.02	1.34
36°	1.11	1.24	0.04	0.55	1.20	0.24	0.99	0.86	1.65	0.10
40°	0.00	0.03	0.03	0.01	0.00	0.00	0.04	0.02	0.24	0.06

Table 7.2 The percentages of the optimal rate of growth (temperature with the highest rate of growth for each isolate used as 100% potential) for each of ten *Metarhizium* spp. isolates at six various temperatures.

	DWR 200	DWR 203	DWR 261	DWR 312	DWR 313	DWR 338	DWR 346	DWR 356	ARSEF 324	ARSEF 1095
20°	62.6%	65.0%	92.4%	81.6%	55.3%	89.8%	70.0%	71.5%	67.8%	78.6%
24°	82.2%	88.1%	***	85.5%	77.4%	97.1%	93.1%	91.0%	86.8%	***
28°	***	***	89.6%	81.4%	***	***	***	***	96.5%	95.7%
32°	64.2%	88.5%	31.0%	***	65.9%	96.7%	87.2%	82.7%	***	30.0%
36°	21.7%	19.4%	0.9%	14.9%	21.4%	6.8%	17.7%	15.0%	41.0%	2.2%
40°	0.0%	0.4%	0.7%	0.3%	0.0%	0.0%	0.8%	0.3%	6.1%	1.2%

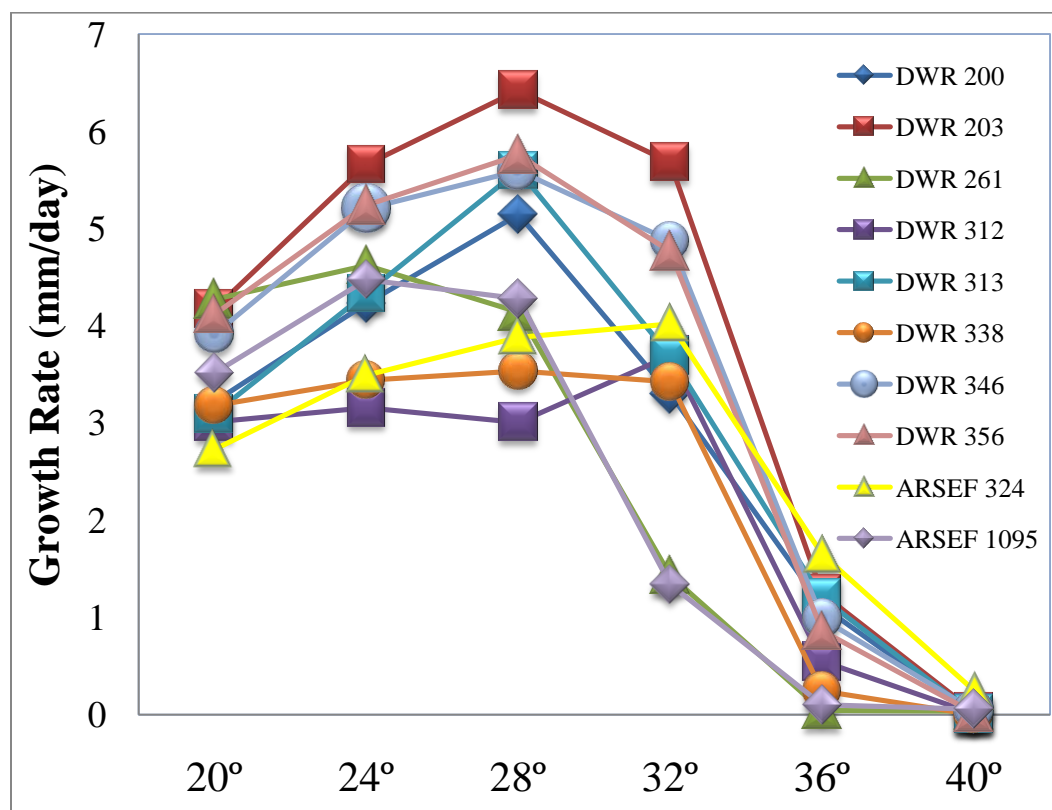


Figure 7.1 The growth rate (mm/day) of ten *Metarhizium* spp. isolates at six different temperatures.

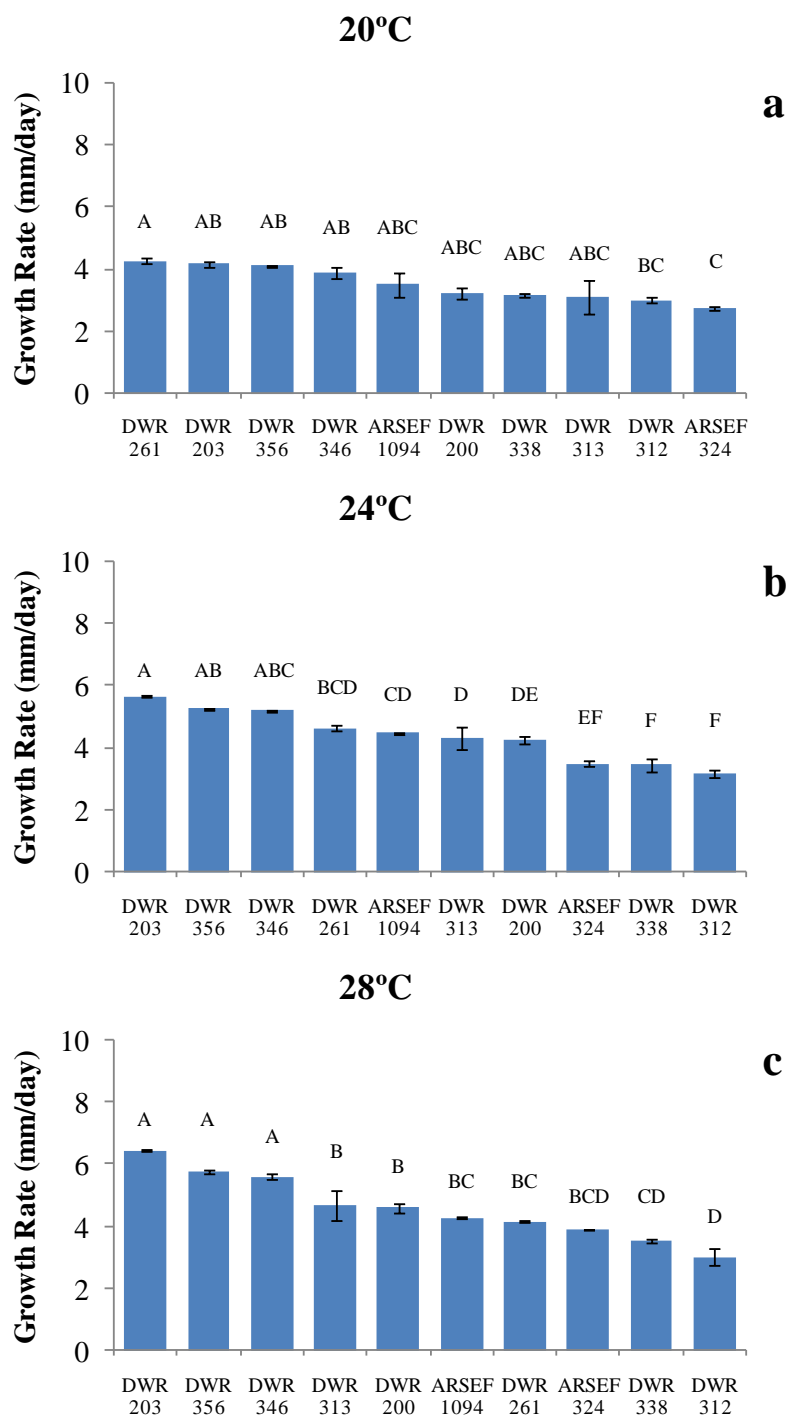


Figure 7.2 The growth rate (mm/day) of ten *Metarhizium* spp. isolates at (a) 20°C, (b) 24°C, and (c) 28°C. The isolates are arranged from greatest rate to lowest at each temperature. Statistically significant differences are designated by letters below the x-axis.

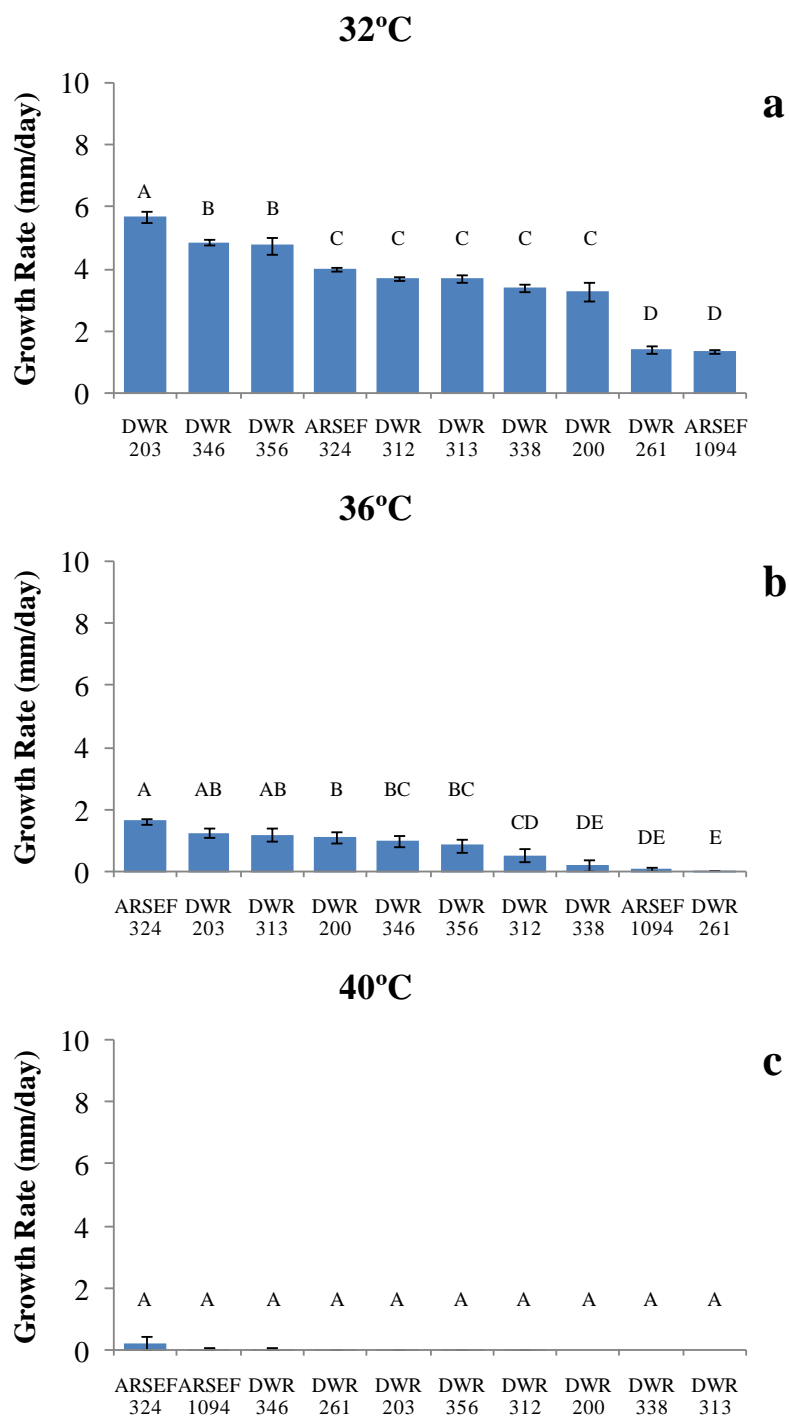


Figure 7.3 The growth rate (mm/day) of ten *Metarhizium* spp. isolates at (a) 32°C, (b) 36°C, and (c) 40°C. The isolates are arranged from greatest rate to lowest at each temperature. Statistically significant differences are designated by letters below the x-axis.

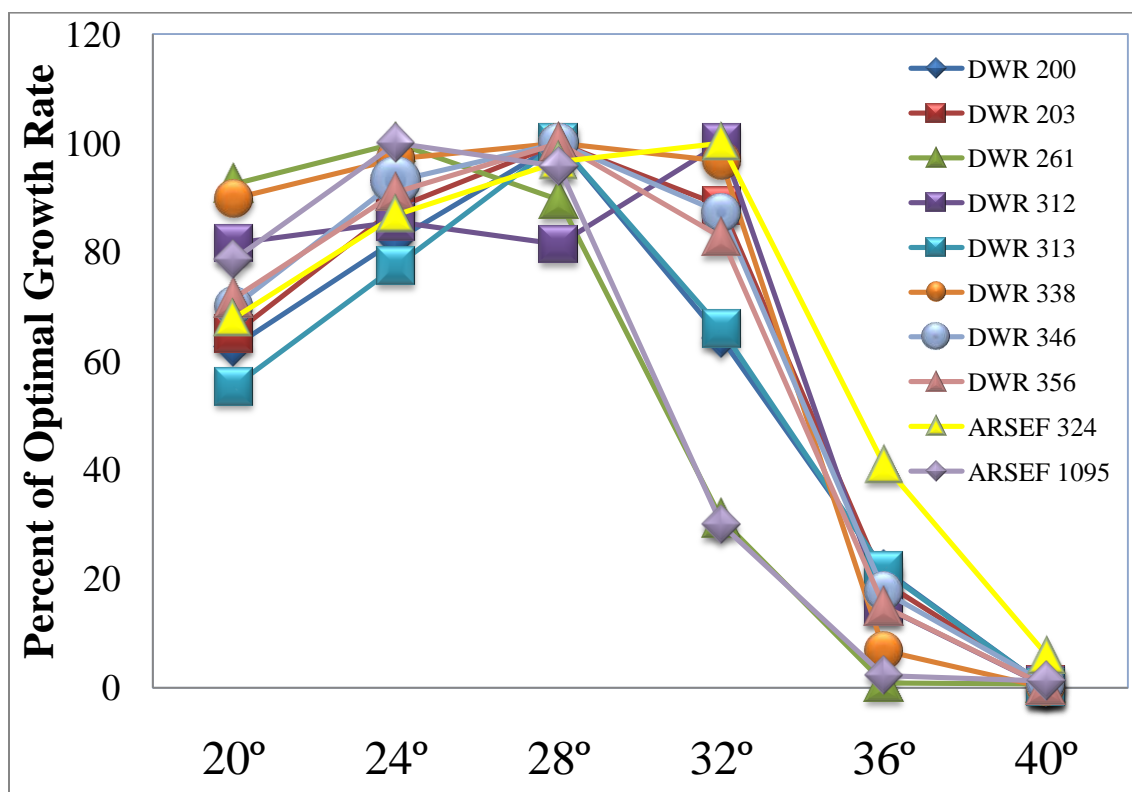


Figure 7.4 The percentages of the optimal rate of growth (temperature with the highest rate of growth for each isolate used as 100% potential) for each of ten *Metarhizium* spp. isolates at six various temperatures.

CHAPTER 8

VARIATION IN CONIDIAL TOLERANCE TO WET HEAT (45°C) OF TEN *METARHIZIUM* SPECIES ISOLATES

Abstract

High heat tolerance is a very important criterion in choosing strains of fungal pathogens with exceptional potential for success as biological control agents. This study investigates the tolerance of ten *Metarhizium* spp. isolates by measuring their ability to germinate after exposure to 45°C wet heat for 2, 4, or 6 hours. ARSEF 324, a *M. acridum* isolate, had more than 90% relative germination after 6 hours exposure. The other species (*M. robertsii*, *M. guizhouense*, and *M. brunneum*) all had poor to no germination after 6 hour exposure. Two- and four-hour heat exposures produced varying results among these isolates; several were moderately tolerant and had greater than 90% germination after 2 hours and more than 50% after 4 hours, while the remainder (including ARSEF 1095, a US commercial strain) had greatly reduced germination following any of the heat treatments. Commonly encountered field conditions dictate that a biological control fungi be somewhat tolerant to high temperatures. Accordingly, selecting isolates based on tolerance to heat will lead to increased field efficacy.

1. Introduction

Achieving desired pest control through an entomopathogenic fungus requires an understanding of both the traits of the pathogen, and the defense mechanisms potentially employed by the target pest. Locusts and grasshoppers have long been recognized as

important pest insects and their control utilizing entomopathogenic fungi from the genus *Metarhizium* has already been implemented with success in several areas of the world, including Africa, Australia and China. *Metarhizium* spp. are currently under investigation in the United States as promising control agents for both grasshoppers and Mormon crickets.

Temperatures above those that allow conidial germination and growth affect cells in different ways. While fungal growth is limited generally around 37°C (Fargues et al., 1997; Luz and Fargues, 1997; Milner, 1997, 2003; Ouedraogo et al., 1997; Thomas and Jenkins, 1997; De Croos and Bidochka, 1999), cell death does not take place unless exposure is prolonged or extremely high temperature are experienced. Depending on the climate and surrounding vegetation, soil can reach temperatures as high as 60-65°C through direct sunlight (Arthurs and Thomas, 2001; Rangel et al., 2005b). Realizing that an isolate's ability to survive high temperature is highly influential to successful biological control, several studies have evaluated spore resistance to high temperatures by determining thermal death points of spores (the lowest temperature at which all spores will be killed within 10 minutes). For most *Metarhizium* spp. isolates, the thermal death point of conidia generally ranges between 45° and 60°C (Zimmermann, 1982; Rangel et al., 2005b). High temperatures can have many effects on conidia, including protein denaturation, membrane disorganization and DNA damage (Crisan, 1973; Setlow, 1995; Setlow and Setlow, 1998). Some spores have elevated tolerance to high temperatures because of either higher amounts of saturated fatty acids, which decreases cellular membrane permeability (Crisan, 1973; Pupin et al., 2000; Guerzoni et al., 2001), or

higher amounts of trehalose and mannitol, which provide protection against denaturation of proteins and membranes (Rangel et al., 2006a).

Infecting spores must not only overcome harsh ambient temperatures, they must also prevail over host defenses. One defensive attribute that Orthopteran pests, including grasshoppers and Mormon crickets, have developed helps them to overcome pathogen infections by altering their behavior to increase body temperature. This defense mechanism is known as “behavioral fever,” and consists of infected orthopterans purposefully using direct sunlight to increase their body temperature above that which a pathogen can grow (Parker, 1982; Kemp, 1986; Whitman, 1987, 1988; Prange and Pinshow, 1994; Thomas and Jenkins, 1997; Arthurs and Thomas, 2001; Blanford and Thomas, 2001; Ouedraogo et al., 2002, 2004; O'Neill and Rolston, 2007). Locusts and grasshoppers have been shown to have a preferred body temperature of 38° to 40°C, and when infected by a pathogen may increase preferred hemolymph temperature to 42°C (Carruthers et al., 1992; Blanford et al., 1998; Blanford and Thomas, 2000; Lomer et al., 2001; Ouedraogo et al., 2003).

For a biological control agent to be successful, it must be able to survive high temperatures and reinitiate growth once cooler temperatures are reached. Accordingly, this study investigates the ability of ten *Metarhizium* spp. isolates to germinate after exposure to 45°C wet heat for 2, 4, or 6 hours.

2. Materials and methods

2.1 Fungal isolates

Ten isolates of *Metarhizium* were used in this study, two of which (ARSEF 324 and ARSEF 1095) were received from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). Bischoff et al. (2009) identified ARSEF 324 as *M. acridum* and ARSEF 1095 as *M. brunneum*. The remaining eight specimens are cataloged in the Donald W. Roberts culture collection (DWR) (Utah State University, Logan, Utah). Fernandes et al. (unpublished) using the methodology of Bischoff et al. (2009), identified DWR 200, DWR 203, DWR 312, and DWR 313 as *M. guizhouense*; DWR 261 as *M. brunneum*; and DWR 338, DWR 346 and DWR 356 as *M. robertsii* (Table 2.1). Stock cultures were maintained at 4°C in test-tube slants of Potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9.

2.2 Conidia suspensions

Conidia of each isolate were produced on 23 ml PDAY medium in 95 x 15 mm polystyrene Petri Dishes. Isolates were allowed to grow for 14 days at 28°C prior to experiment initiation. Conidia were harvested using a microbiological loop and suspended in 0.01% Tween 80 (Sigma-Aldrich). Suspensions were vortexed and passed through an Isopore™ Membrane filter (25-mm diameter, 8-μl pore size, Millipore, Billerica, MA, USA) to remove hyphae, spore aggregates and germinated conidia. Suspensions were used immediately.

2.3 Wet heat exposure

For each suspension, 2 ml was transferred to a screw-cap glass tube (16 x 125 mm, Pirex[®], Corning[®], NY, USA) and placed in a stirred water-bath at 45°C for the following time intervals: 0 (control) 2, 4, and 6 hours. Germination capability was assessed by inoculating 20 µl of the treated suspension onto 4 ml PDAY plates with 0.002% benomyl (active ingredient) (Hi-Yield Chemical Company, Bonham, TX) (Milner et al., 1991) in 35 x 10 mm Petri plates. Plates were incubated at 28 ± 1 °C for 48 hours. Relative percent germination was calculated by comparison with control plates. Percent germination was determined by observing, after 48h incubation, 300 conidia under 400X magnifications for the presence or absence of visible germ tubes.

2.4 Statistical analysis

The effect of heat treatment on spore survival was assessed using a one-way ANOVA with complete block design. The assumptions of normality and homogeneity of variance were met without transformations. Mean comparisons among treatments were unadjusted for family-wise Type I error using the LSD method to maximize power to detect potential differences; *P*-values greater than 0.05 were considered as significant. Data analyses were generated using the MIXED procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows.

3. Results

Variability in responses to heat-treatment times was observed among the isolates of *Metarhizium*. ARSEF 324, the only *M. acridum* used in this experiment, exhibited

stellar tolerance to high heat, while the other *Metarhizium* isolates were markedly more sensitive, especially as length of exposure increased.

Two-hour exposures did not result in a major amount of variability among isolates ARSEF 324, DWR 356, DWR 203 and DWR 346. These isolates all had nearly 100% relative germination, and constitute a statistically unique group (Figure 8.1). DWR 312, DWR 338, DWR 200 and DWR 313 averaged 70.4, 65.1, 61.0, and 58.2 percent relative germination respectively, and were all statistically similar. DWR 261, isolated from Alaskan soil, and commercial isolates ARSEF 1095 were the most susceptible to heat; after two-hour treatment only 15.4 and 37.33% of the conidia germinated, respectively.

Four-hour heat treatment was enough to seriously inhibit germination for a majority of *Metarhizium* isolates, including DWR 200, DWR 312, DWR 313, DWR 338, ARSEF 1095, and DWR 261, which had only germination levels of 13.1, 10.1, 9.5, 2.3, 0.4, and 0.2%, respectively, and these levels were not statistically different (Figure 8.1). DWR 356 and DWR 203 both had decent germination after 4 hours exposure to heat, with 67.5 and 62.8%, respectively; while DWR 346 had less than 50% germination after 4 hours exposure (43.3%). ARSEF 324 was seemingly unaffected by the treatment, yielding 96.6% relative germination.

Exposure to 45°C for 6 hours had a negligible effect on *M. acridum* isolate ARSEF 324 (92.4% relative germination), but was detrimental to the other isolates. Only DWR 203 was able to achieve above 10% relative germination (16.43%), while DWR 346 and DWR 356 had 5.1 and 4.8%, respectively. The remainder of the isolates (DWR

313, DWR 200, DWR 312, DWR261, ARSEF 1095, and DWR 338) produced insignificant germination (less than 2%) after this prolonged treatment.

4. Discussion

The sensitivity to 45°C seen among the *M. robertsii*, *M. guizhouense* and *M. brunneum* isolates is disheartening. While 6 hours at 45°C might be extreme for field situations, 4 hours is plausible, and 2 hours exposure to 45°C is realistic for some desert settings. ARSEF 1095, a commercialized USA isolate, lost more than 60% of its viability after just 2 hours exposure to 45°C, and 4 hours essentially killed all the spores. An isolate with this level of sensitivity should not be considered for field use in regions where the ambient temperature is frequently elevated.

Clearly the isolate superior to all others in this study as far as high temperature tolerance is ARSEF 324, an isolate obtained from an orthopteran host in Australia, and currently used there for control of locusts. Other *M. acridum* isolates also are known to be extremely tolerant to high temperatures. In fact, Fernandes et al. (2010) suggested that the consistency of heat tolerance among *M. acridum* isolates could be used as a preliminary identification tool for isolates of this species. Rangel et al. (2005b) also found ARSEF 324 to be highly heat tolerant, having 80% germination after exposure to 49°C for 2 hours. It is likely that the tolerance to heat exhibited by *M. acridum* isolates evolved congruently with its specificity for orthopteran insects and their affinity for warm temperate regions.

Further attestation of the need for heat-tolerant entomopathogenic fungi in warm climates is accentuated by the defensive mechanism of some insects known as behavioral

favor. Locust and grasshoppers have been shown to have a preferred body temperature of 38° to 40°C and when infected with a pathogen may increase their preferred hemolymph temperature to 42°C (Carruthers et al., 1992; Blanford et al., 1998; Blanford and Thomas, 2000; Lomer et al., 2001; Ouedraogo et al., 2003). For example, Elliot et al. (2002) observed that when the desert locust *Schistocerca gregaria* was infected in the laboratory with *M. acridum* (IMI33019), no deaths occurred within 53 days if the insects were allowed to thermoregulate reaching body temperatures as high as 44.3°C, but the insects died within three days after the thermal treatment ceased. In addition, Turnbow (Turnbow, 1998) showed that Mormon crickets (MC) do in fact exhibit a slight, yet statistically significant, behavioral fever (~0.5°C) when infected with *Beauveria bassiana*. Turnbow also noted that the preferred temperature of MC, near or above 35°C, may be sufficient to impede or block fungal infection.

Heat-tolerant fungi have several mechanisms that allow them to live in harsh environments. One important mechanism is the endogenous accumulation of trehalose and mannitol in fungal mycelium and spores. Trehalose protects cells against several stress factors by stabilizing proteins in their native state and by preserving the integrity of membranes (Singer and Lindquist, 1998). Mannitol protects cells by scavenging toxic oxygen intermediates from stresses (Noventa-Jordao et al., 1999). Cells accumulate trehalose notably in response to stresses, including heat shock (Eleutherio et al., 1993). Rangel et al. (2008b) observed that conidia produced on heat-shocked mycelium were more tolerant to UV and to heat exposure. Furthermore, Rangel observed that conidia

produced on nutrient-deprived media had a significantly higher level of mannitol and trehalose.

In summary, *M. acridum* isolate ARSEF 324 tolerated exposure to 45°C significantly better than any of the other (non-acridum) isolates. After 4 hours exposure, DWR 356, and DWR 203 were the only non *M. acridum* isolates to have greater than 50% germination. Commonly encountered field conditions dictate that a biological control fungus be somewhat tolerant of high temperatures. Accordingly, selecting isolates based on tolerance to heat may afford biocontrol agents with high field efficacy.

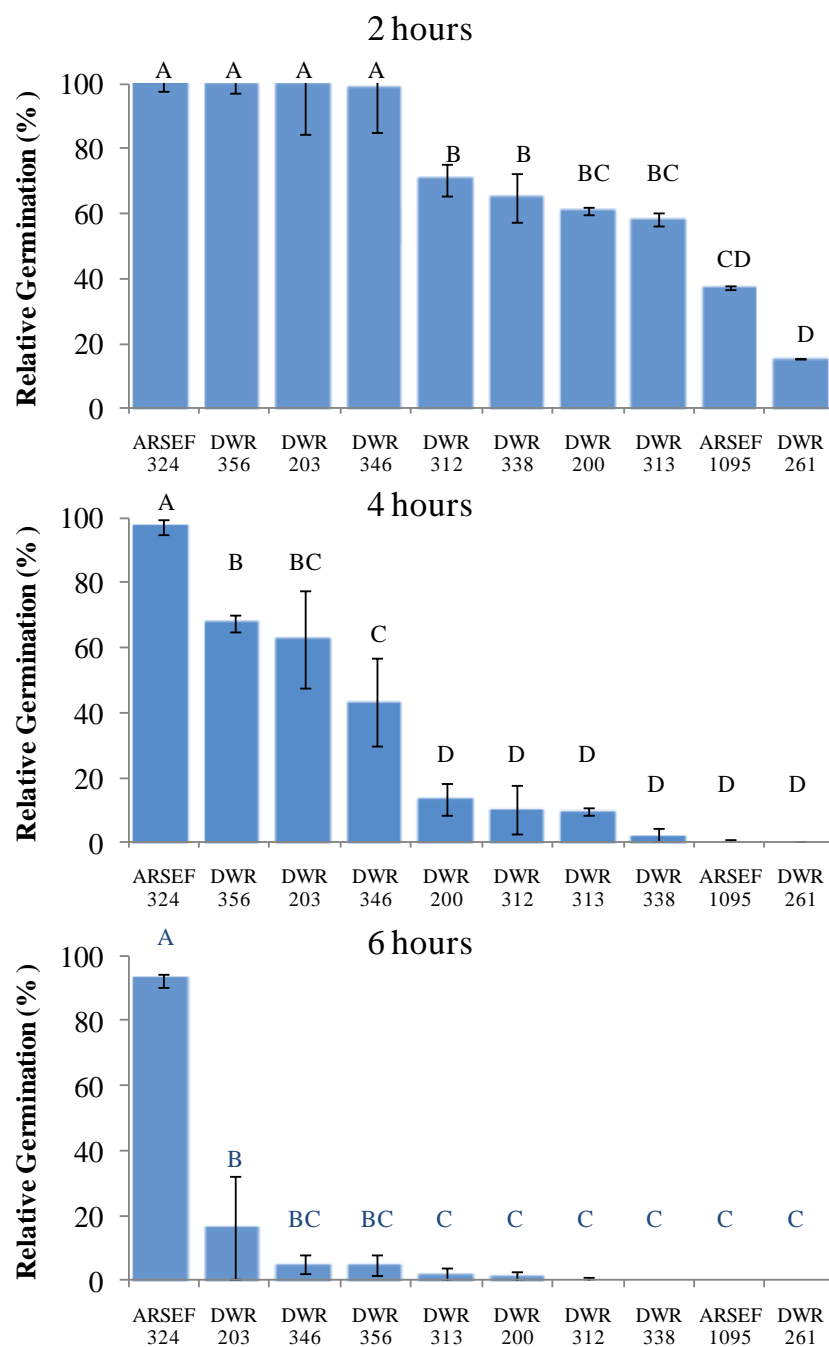


Figure 8.1 Relative percent germination of conidia of ten *Metarhizium* spp. isolates after exposure to 45°C wet heat for 2, 4, or 6 hours. Data represents averages of 3 trials conducted on separate days with new conidial cultures of each isolate used each day. Variation among replicates is indicated by standard error bars. Statistically significant differences are represented by letter designations below the x-axis.

CHAPTER 9

HEAT-INDUCED POST-STRESS GROWTH DELAY: A BIOLOGICAL TRAIT OF MANY *METARHIZIUM* ISOLATES THAT MAY REDUCE FIELD EFFICACY

Abstract

Many pest insects occur in temperate regions that commonly have fluctuating temperatures that can range from 0° to over 40°C within a single day. It has generally been assumed that a fungal isolate is unlikely to infect insects at temperatures above its growth threshold; but, as long as the elevated temperature only inhibits (does not kill) the fungus, disease development will proceed at night and during cooler periods of each day. A detailed study of ten *Metarhizium* spp. isolates made clear, however, that for several of the isolates, mere survival after exposure to high temperatures may not be sufficient to allow infection in field situations where daily temperatures repeatedly exceeded 40°C. Some *Metarhizium* isolates were remained in stasis for least 24 hours post heat exposure prior to resuming growth. This phenomenon is labeled “heat-induced post-stress growth delay” (PSGD) in this study. Heat-induced PSGD was evaluated by exposing three-day-old colonies on agar media to 40°C for 4 or 8 hours followed by 20 or 16 hours at 28°C, respectively, for 3 days; after which all treatments were held at constant 28°C for 7 days. Growth rates (mm/d) during and following heat treatments were compared to control plates (constant 28°C) and to plates with intermittent growth stoppage by cold treatment (4 or 8h at 5°C per day). While all isolates survived 3 days of cycled heat treatment and resumed normal growth afterwards, some isolates were considerably more affected by

heat-cycling than others. Two of the faster growing isolates, DWR 346 and DWR 356, had their growth rates reduced by 18.6 and 14.2% after 4h/d, and 87.2 and 79.9% with 8h/d heat treatment, respectively, during the three days of heat treatment. Conversely, isolates that had relatively low growth rates at constant 28°C (i.e., ARSEF 324, DWR 312, and DWR 338) resumed normal growth rates between heat pulses. The growth-rate reduction of isolates with high heat-induced PSGD potentially could be devastating during field applications, rendering these isolates virtually useless after a series of hot days. An effort to test in the laboratory this expectation of reduced efficiency (insect mortality) with repeated (daily) temporary high temperature exposure was conducted. Three *Metarhizium* isolates were bioassayed using *Galleria melonella*, a high-temperature-tolerant insect. The insect larvae were exposed to conidial suspensions, and placed at temperature regimes matching those utilized for the agar-medium colony-growth study. Insect mortality was recorded daily. Both cold and hot temperature regimes delayed the time to death, but the high temperature regimes drastically increased the time it took for the insects to die. The only significant differences among the isolates were with the daily eight-hour heat treatments, i.e., DWR 203 killed significantly faster than DWR 312. Fluctuating temperatures are likely to be a factor in most pest-insect habitats; therefore, in addition to evaluating spore survival, PSGD should be a primary consideration for field-appropriate isolate selection.

1. Introduction

Problematic outbreaks of orthopteran pests usually occur in dry, warm climates, such as in Africa, Australia, China, and the Western United States (Faria and Wraight, 2007). In these temperate and desert regions, pest outbreaks often occur during periods when temperatures are fluctuating and can range from 0° to over 40°C within a single day. Regrettably, the high day-time temperatures are often a major hindrance for biological control agents. It is generally assumed that a fungal isolate is unlikely to infect insects at temperatures above its growth threshold, but as long as the fungus is not killed, infection and disease development will proceed during cooler periods of the day (Rangel et al., unpublished). Consequently, temperature studies often focus on spore survival, including thermal death points or relative germination after thermal exposure (Arthurs and Thomas, 2001; Rangel et al., 2005; Zimmermann, 1982). This information is useful in determining which isolates have a propensity to survive potentially high field-temperatures; however, these studies neglect any non-lethal effect resulting from the exposure.

Rangel (2006) reviewed several methods implemented by fungi to survive exposure to intense heat stress, including: 1) reducing membrane fluidity and disintegration by increasing saturated fatty acids in the cell membrane (Steels et al., 1994; Swan and Watson, 1999; Guerzoni et al., 2001); 2) high levels of cell wall-associated melanin or cytoplasmic carotenoids for photo-protection (Butler and Day, 1998; Henson et al., 1999; Arcangeli and Cannistraro, 2000; Zhdanova et al., 2000); 3) producing antioxidant enzymes, including superoxide dismutase and catalase (Kapoor and

Sveenivasan, 1988; Kapoor et al., 1990; Noventa-Jordao et al., 1999; Franca et al., 2007); 4) increasing nucleic acid CG content to provide more thermostability (Rothschild and Mancinelli, 2001); 5) DNA repair mechanisms (Griffiths et al., 1998); 6) increasing levels of trehalose and mannitol (Sanchez et al., 1992; Singer and Lindquist, 1998); and 7) possessing heat shock proteins that are involved in folding newly synthesized proteins or in the repair of misfolded/aggregated proteins (Iwahashi et al., 1998; Rensing et al., 1998). These techniques are useful in helping a spore survive exposure to high temperatures, but with some fungal isolates cell-damage repairs and/ or resource-allocation for stress protection may delay fungal growth.

If significant growth delay due to heat-induced post-stress trauma is experienced with an isolate, a cyclic climate with high heat extremes may not provide opportunity that an entomopathogen to infect a host or fully develop, thus rendering it ineffective as a biological control agent. Accordingly, this study evaluates the effects of temperature stress on the colony growth of ten fungal isolates on agar medium and on virulence of three fungal isolates to *Galleria melonella*.

2. Materials and methods

2.1 Field temperatures

Field temperatures were measured using artificial Mormon crickets as described by Lactin and Johnson (1998). Artificial MC were constructed by filling a 1.5 ml plastic centrifuge tube (Fisher Scientific) with soy sauce (ConAgra Brands, Inc., Irvine CA). A hypodermic needle (21, Monoject 200, Fisher Scientific) containing a Betatherm thermistor (10K3MCDZ, Measurement Specialties, Hampton VA) was inserted through

the centrifuge-tube wall and sealed in place with thermal conducting cement.

Temperatures were recorded hourly on an H12 Hobo data logger with external data ports (Onset, Bourne, MA). In 2008 surrogate-insect temperature monitors were placed in a field planted with red winter wheat in Logan UT (41°46'6.99"N, 111°49'22.38"W). In 2009, recorders were placed in a field planted with alfalfa and mixed grasses in Logan, UT (41°45'37.35"N, 111°48'42.94"W).

2.2 Fungal isolates

Ten isolates of *Metarhizium* were used in this study, two of which (ARSEF 324 and ARSEF 1095) were received from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). Bischoff et al. (2009) identified ARSEF 324 as *M. acridum* and ARSEF 1095 as *M. brunneum*. The remaining eight specimens are cataloged in the Donald W. Roberts culture collection (DWR) (Utah State University, Logan, Utah). Fernandes et al. (unpublished) using the methodology of Bischoff et al. (2009), identified DWR 200, DWR 203, DWR 312, and DWR 313 as *M. guizhouense*; DWR 261 as *M. brunneum*; and DWR 338, DWR 346 and DWR 356 as *M. robertsii* (Table 2.1). Stock cultures were maintained at 4°C in test-tube slants of Potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9.

2.3 Vegetative growth treatments

Conidia of each isolate were produced on 23 ml PDAY medium in 95 x 15 mm polystyrene Petri Dishes. Isolates were allowed to grow for 14 days at 28°C prior to

experiment initiation. Conidia were harvested using a microbiological loop and suspended in 0.01% Tween 80. Suspensions were used immediately.

For each of three trials, 2 μ l of suspension was placed in the center of a Petri dish (23 ml PDAY 95 x 15 mm polystyrene Petri Dishes). Fungal colonies were allowed to develop for 72 hours, and then moved to their respective treatments. For each isolate, individual plates were subjected to the following treatments: “Control,” plates allowed to remain at a constant 28 \pm 1 $^{\circ}$ C; “Cold treatment 4 hours,” plates placed at 5 \pm 1 $^{\circ}$ C for 4 hours, then moved to 28 \pm 1 $^{\circ}$ C for 20 hours; “Cold treatment 8 hours,” plates placed at 5 \pm 1 $^{\circ}$ C for 8 hours, then moved to 28 \pm 1 $^{\circ}$ C for 16 hours; “Heat treatment 4 hours,” plates placed at 40 \pm 1 $^{\circ}$ C for 4 hours, then moved to 28 \pm 1 $^{\circ}$ C for 20 hours; “Heat treatment 8 hours,” plates placed at 40 \pm 1 $^{\circ}$ C for 8 hours, then moved to 28 \pm 1 $^{\circ}$ C for 16 hours. Treatments were repeated for three 24 hour cycles. After 3 days’ treatment, all plates were returned to constant 28 \pm 1 $^{\circ}$ C and held for 7 days (Fig. 9.1). Colony growth was determined by measuring (mm) across two diameters for each colony every 24 hours using a carbon fiber composites digital caliper with 0.1 mm (\pm 0.2) resolution (Fisher Scientific). The measurement made immediately before the first treatment was subtracted from each subsequent measurement to establish a zero point for each isolate/treatment combination.

2.4 Bioassay

A virulence assay was conducted while maintaining the daily temperature cycling regimes described above (2.3). Three fungal isolates were selected based on the levels of heat-induced PSGD: low= DWR 312, Moderate = DWR 203 and high = DWR 346.

Conidia used for assays were harvested from 14 day old cultures grown on PDAY plates (polystyrene, Petri dishes, 95x 15mm, Fisherbrand Pittsburg, PA, USA) incubated in the dark at $28\pm 1^{\circ}\text{C}$. Harvested conidia were suspended in 40 ml of 0.01% Tween 80 solution in 50-ml tubes (Modified polystyrene, Corning, Coning, NY, USA). Each suspension was vortexed and conidial concentrations were estimated by hemocytometer counts. Conidial suspensions with a concentration of 1×10^7 conidia ml^{-1} was prepared for each isolate. Suspensions were used immediately for bioassay experimentation.

Fresh commercially produced *Galleria melonella* larvae were used as the host insect. Ten insects were placed in a 60×15 mm polystyrene Petri Dishes lined with a 5.5cm P4 filter paper (Fisherbrand; Porosity: Medium–Fine, Flow rate: Slow). The filter paper was moistened with 50 μl of distilled H_2O to establish high humidity ($\sim 100\%$ RH). The dorsal region of each larva was inoculated with 10 μl of either a fungal suspension or 0.01% tween (for non-treated control insects). For each temperature/isolate combination, thirty insects in 3 Petri dishes were used. The temperature treatments were: constant-temperature treatment, maintained at $28\pm 1^{\circ}\text{C}$ continually; cold treatment for 4 hours, insects exposed to $5\pm 1^{\circ}\text{C}$ for 4 hours then moved to $28\pm 1^{\circ}\text{C}$ for 20 hours; cold treatment for 8 hours, insects exposed to $5\pm 1^{\circ}\text{C}$ for 8 hours then moved to $28\pm 1^{\circ}\text{C}$ for 16 hours; heat treatment for four hours, insects exposed to $40\pm 1^{\circ}\text{C}$ for 4 hours then moved to $28\pm 1^{\circ}\text{C}$ for 20 hours; and heat treatment for 8 hours, insects exposed to $40\pm 1^{\circ}\text{C}$ for 8 hours then moved to $28\pm 1^{\circ}\text{C}$ for 16 hours. Treatments were repeated for fifteen 24 hour cycles. Insect mortality was assessed daily.

2.5 Statistical analysis

The rates of growth, during the treatment period, of each isolate with each temperature treatment were analyzed using a one-way ANOVA with complete block design. The assumptions of normality and homogeneity of variance were met without transformations. Mean comparisons among treatments were unadjusted for family-wise Type I error rate using the Tukey-Kramer method to maximize power to detect potential differences; *P*-values less than 0.05 were considered as significant. Data analyses were generated using the MIXED procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows.

The effect of temperature regime and fungal treatments on *Galleria melonella* was evaluated by survival analysis (Kaplan and Meier, 1958). Insects surviving beyond day 14 were considered censored. LT₅₀s (time [days] to achieve 50% mortality) were generated based on the Kaplan-Meier model using the LIFETEST procedure in SAS. Statistically significant differences between isolates within a temperature regime were calculated based on the Cox Proportional Hazards Model using the PHREG procedure in SAS. *P*-values less than 0.008 (0.05 divided by six pairwise comparisons) were considered as significant, and statistically significant differences between temperature regimes within an isolate were also calculated using the same model with *P*-values less than 0.005 (0.05 divided by ten pairwise comparisons) being considered significant.

3. Results

3.1 *Field temperature*

From June 13 to July 27, 2008, field temperatures in artificial MC ranged from -4.14° to 49.95°C (Figure 9.2). For 42 of the 45 days measured, field temperatures were above 40°C, totaling 230 hours, and averaged greater than 5 hours each day. For several days in July, temperatures reached above 40°C for more than 8 hours, and one day it was above 40°C for more than 9 hours (Figure 9.2 b & c).

From June 13 to July 27, 2009, field temperatures ranged from 7.04° to 43.98°C. For 19 of the 45 days temperatures reached above 40°C, totaling only 50 hours total time above 40°C. On July 17, 2009, the warmest day recorded, the temperature was above 40°C for more than 5 hours.

3.2 *Cold treatment*

Control fungal colonies kept at constant 28°C and measured every 24 hour for 72 hours grew at an average rate 17% faster than colonies treated to 4 hours of 5°C during every 24-hour period (Figure 9.3). Proportionately, nearly all colonies treated with 8 hours of cold (16h at 28°C) for 3 days, grew 33% slower than colonies of the same isolate with constant 28°C incubation. Isolate DWR 313 was the only major exception, achieving a rate higher than expected based on controls estimates. The data indicate that there was likely a error in the experimental set up involving DWR 313, as the control achieved lower rate than previously observed at that constant temperature (3.88 compared to 5.60 mm/day), and the growth was non-linear over the course of the experiment (Table 9.1).

Growth rates after exposure to 5°C for 4 hours ranged from 5.52 mm/day (DWR 203) to 2.62 mm/day (DWR 338); while 8 hour cold treatment growth ranged from 3.92 mm/day (DWR 203) to 1.92 mm/day (DWR 338) (Table 9.1).

3.3 Heat treatment

The effect of the heat treatment varied between isolates. Isolates DWR 312, DWR 338 and ARSEF 324 were not significantly affected by the heat treatment, i.e., their growth rates were similar to those of corresponding cold-treated plates. Isolates DWR 200, DWR 203, DWR 261 and ARSEF 1095 were all similarly affected by the heat treatment, viz., 8 hours treatment had nearly double the effect of 4 hours treatment. DWR 313 seemed to be affected by the heat treatment; but, due to inconsistencies in the control growth, the level of effect was not quantifiable. DWR 346 and DWR 356 were both clearly negatively affected by the two heat treatments, with greater affect by 8 hours exposure than 4 hours (Figure 9.4). After 8 hours heat exposure, growth rates for DWR 261, DWR 346, and DWR 356, dropped to less than 1mm/day, i.e., virtually stopping growth (Table 9.1).

Average growth rates for seven days after the 4-hour treatments of 40°C heat-exposures ranged from 5.08 mm/day (DWR 203) to 2.59 mm/day (DWR 261). While rates after the 8-hour treatments ranged from 2.95 mm/day (DWR 203) to 0.50 mm/day (DWR 346) (Table 9.1).

3.4 Bioassay

Galleria melonella larvae are highly susceptible to infection by *Metarhizium*; and all the three isolates tested caused 100% mortality within 5 days of treatment at the constant 28°C temperature regime (Figure 9.5). With all treatments (both the temperature regimes and the three fungal isolates), had significantly higher mortality than the controls (Table 9.2). Survival analysis proved an efficient method to make comparisons among the temperature regimes and between the three isolates. All three isolates achieved 50% mortality within 3 days at the constant temperature. Treatments at 5°C for four or eight hours every 24 hour period increased the time it took to reach 50% mortality (LT₅₀) to approximately 4 and 5 day, respectively, for all the fungal isolates (Figure 9.6). The LT₅₀s for the temperature regimes with heat were increased significantly. Both four hour and eight hours at 40°C heat treatments each day increased the survival time by statistically significant amounts (averaging 6.3 and 12.3 d, respectively). Variation between the isolates at each temperature regime was less obvious, and no statistically significant differences were detected between the isolates at each temperature regime, except for eight hours of 40°C each day. At this temperature regime DWR 203 had a LT₅₀ of 11 days, while DWR 312 and DWR 346 had LT₅₀s of 14 and 12 days, respectively. Only DWR 203 and DWR 312 had significantly different LT₅₀ (p=0.0019).

4. Discussion

Field temperatures recorded by the artificial (surrogate) MC in Logan, Utah, frequently dropped to, or even below 5°C during the summer of 2008; but during the same dates in 2009 temperatures less frequently reached below, 10°C (Figure 9.2). This

study observed that colony growth ceases during exposure to cold temperatures (5°C), thus providing pest insects with a reprieve from infecting fungal pathogens. Fernandes et al. (2010) also observed that *M. anisopliae* s.l. and *M. acridum* isolates had no, or only minimal, conidial germination at 5°C during 15 days; but many isolates did germinate during 15 days at 10°C. The temperature differences recorded between 2008 and 2009 possibly resulted from field vegetation. The field planted with red winter wheat in 2008 provided sparse vegetation and greater exposure to direct sunlight for long periods of the day; while the alfalfa field in 2009 was densely vegetated, providing the surrogate-insect temperature monitors with less direct sunlight and protection from chilling winds. Cycling field temperatures reduce the length of time per day that fungi are exposed to permissible growth temperatures. After a cold or hot period which stops fungal activity, an isolate's ability to begin growing immediately is important for disease development. After returning from cold temperatures to optimal growth temperatures, all isolates in this study resumed normal growth rates, indicating that moderately cold temperatures that cease fungal growth during exposure to cold do not induce a post-stress growth delay (PSGD).

High temperatures have been shown to be extremely hazardous to the health of a *Metarhizium* spores (Rangel et al., 2005b). The majority of thermal studies involving entomopathogens have focused primarily on survival, i.e., evaluating high-temperature endurance and characteristics which enhance survival. In one such study, Zimmermann (1982) noted a PSGD in *Metarhizium* spores exposed to 45°C for 30 min. These spores had low germination after 24 hours, but after 48 hours, germination increased

significantly and approached the observed germination of unheated spores after 24 hours. Another study observed that the vigor of a colony's growth after heat exposure was dependent on the intensity of the stress treatment (i.e., temperature and time) (Liu et al., 2009). Unfortunately, this phenomenon is often observed, but seldom commented upon in detail.

UV induced PSGD has also been observed by several authors in surviving *Metarhizium* spores exposed to UV-B radiation. Alves et al. (1998) observed that spores treated with ultraviolet radiation exhibited severely delayed germination. Braga et al. (2001a; 2001b, 2001c) also noted delayed germination in surviving spores; and stated that the delay was in direct proportion to the treatment dose in all isolates of tested *Metarhizium* exposed to full-spectrum sunlight, UV-B and UV-A. The reasons for this delay in germination of surviving spores exposed to UV are unclear, but slower growth may possibly be due to nucleic-acid and protein damage, a defensive response in conidia, or diverted energy to repair cell damage.

This study clearly demonstrated that colony exposure to 40°C can cause hyphal growth delay in some isolates, while having little effect on others. An isolate's ability to survive exposure to environmental stress is highly important to successful pest control. Similarly, PSGD potentially can be as pernicious to a control program as is conidia mortality. If an isolate is unable to expeditiously recover from high heat exposure, its mere survival will be nullified by its inability to cause disease development. Several authors have observed a positive correlation between rapid germination and hypervirulence of *Metarhizium* strains (Al-Aidroos and Roberts, 1978; Al-Aidroos and

Seifert, 1980; Hassan et al., 1989; Samuels et al., 1989). Likewise, delay of growth following stress exposure would be expected to reduce virulence.

Isolates DWR 312 and ARSEF 324 were not affected by either 4 or 8 hours of heat exposure, as evidenced by their maintaining growth rates similar to those obtained with the cold temperature regimes. Interestingly, of the isolates tested, these had the lowest rates of growth at 28°C (Table 9.1). Perhaps their relatively slow natural colony expansion results from the utilizing of resource and energies to protect against, and repair damage caused by, environmental stresses. ARSEF 324 is a *M. acridum* isolate and this species, in general, is distinguished by its exceptional heat tolerance capabilities (Fernandes et al., 2010). Furthermore, several studies have evaluated the tolerance of ARSEF 324 to heat and found it to be exceptionally tolerant to heat (Rangel et al., 2005b). The lack of a PSGD in these isolates suggests a correlation between survival and growth delay avoidance, although further studies are needed to confirm this.

Unexpectedly, the two isolates whose growth was most affected by heat exposure were DWR 346 and DWR 356, both of which have high rates of growth (5.61 and 5.66 mm/day, respectively) at 28°C compared to other isolates. Four hours per day of 40°C exposure reduced their growth rates by 18.6 and 14.2%, and 8 hours per day exposure reduced rates 87.2 and 79.9%, respectively. This intense rate reduction could be devastating during field applications, and render these isolates virtually useless after several warm days. In this study, DWR 203 was the only isolate to have a faster growth rate at 28°C (6.71 mm/day) than DWR 346 and DWR 356. DWR 203 was affected by the transient heat exposure and lost nearly 1mm growth per day after 8 hours of exposure

(0.5 mm/day after four hours exposure) when compared to the cold or control treatments. However, even after this delay in growth, DWR 203 was able to rebound immediately with a high growth rate of 3.92 mm/day, which was faster than the regular growth rates with no stress exposure of the isolates that did not have an observable PSGD (DWR 312 and ARSEF 324). Accordingly, if growth rate is directly correlated with high virulence, DWR 203 would continue to be an effective agent, despite its PSGD.

For the bioassay, three isolates were selected based on their levels of growth-rate reduction observed after exposure to 40°C for 4 or 8 hours per day. DWR 312 showed no growth rate reduction, DWR 203 showed a moderate rate reduction, and DWR 346 demonstrated a high rate reduction. Due to its specificity toward orthopteran insects, ARSEF 324 was not included in this bioassay study using a lepidopteran, *G. melonella*. Differences between the low temperature regimes and the high temperature regimes were obvious; exposure to high temperature for either four or 8 hours each day significantly increased the time required to cause mortality for all the isolates. In fact, 100% insect mortality was not observed by the end of the experiment (14 days) with any of the isolates exposed to the 40°C for 8-hour regime (Figure 9.5). Nevertheless, DWR 346 was not statically slower at causing mortality than either DWR 312 or DWR 203 (Table 9.2). DWR 203, with its high growth rate was able to cause mortality faster than the slow growing DWR 312 at the eight hour 40°C temperature regime. Interestingly, while not all insects were killed by the fungal treatments with the daily eight hour high-temperature regime, those that survived showed severe signs of infection (i.e. cuticle melonization)

and altered behavior; and in all likelihood, these insects while technically alive, would not be a pest concern.

Environmental stress can be a debilitating factor strongly influencing the success or failure of pest control programs utilizing fungal entomopathogens. Whether it is heat, UV, nutrient deprivation, dehydration, or some other unknown factor inducing the stress, the post-stress results are likely to be varied. A fungal isolate that survives, but is unable to infect target pests due to delayed growth, is of little use in insect control programs. Fluctuating temperatures are likely to be a primary concern in most pest-insect habitats; therefore, in addition to evaluating spore survival, heat-induced PSGD should be a primary consideration for field-appropriate isolate selection.

Table 9.1 The rate of growth (mm/day) for ten *Metarhizium* spp. isolates exposed to one of five temperature treatments for three days. The growth change (%) due to heat was calculated by dividing the growth rate of heat-treated colonies by the growth rates of cold treated colonies of the same isolate.

Isolate	Control 28°C	5°C Treatment		40°C Treatment		Growth change (%) due to heat ^(b)	
		4 hours/d	8 hours/d	4 hours/d	8 hours/d	4 hours/d	8 hours/d
DWR 200	5.33	5.49	3.71	5.02	2.87	8.6%	22.6%
DWR 203	6.71	5.52	3.92	5.08	2.95	8.0%	24.7%
DWR 261	4.47	3.96	2.99	2.59	0.65	34.6%	78.3%
DWR 312	2.60	3.10	2.66	3.17	2.81	-2.3%	-5.6%
DWR 313	3.88	5.50	3.74	4.74	2.89	13.8%	22.7%
DWR 338	2.87	2.62	1.92	2.64	1.61	-0.8%	16.1%
DWR 346	5.61	5.05	3.90	4.11	0.50	18.6%	87.2%
DWR 356	5.66	4.86	3.64	4.17	0.73	14.2%	79.9%
ARSEF 324	3.61	3.09	2.32	3.10	2.25	-0.3%	3.0%
ARSEF 1095	4.71	3.86	2.93	3.00	0.91	22.3%	68.9%

Table 9.2 The time (days) required to reach 50 percent mortality (LT₅₀).

Temperature regime	DWR 203	DWR 312	DWR 346	Control
Constant	3 A, a	3 A, a	3 A, a	* B, ab
4h at 5°C	4 A, b	4 A, a	4 A, b	* B, bc
8h at 5°C	5 A, b	5 A, b	6 A, c	* B, c
4h at 40°C	7 A, c	7 A, c	5 A, d	* B, abc
8h at 40°C	11 A, d	14 B, d	12 AB, e	* C, a

Statistical significant groupings

* LT₅₀ greater than 14 days

Uppercase letter indicates relationship between isolates at each temperature (rows)

Lowercase letters indicate relationship of temperature regimes for each isolate (columns)

PSGD treatment plan

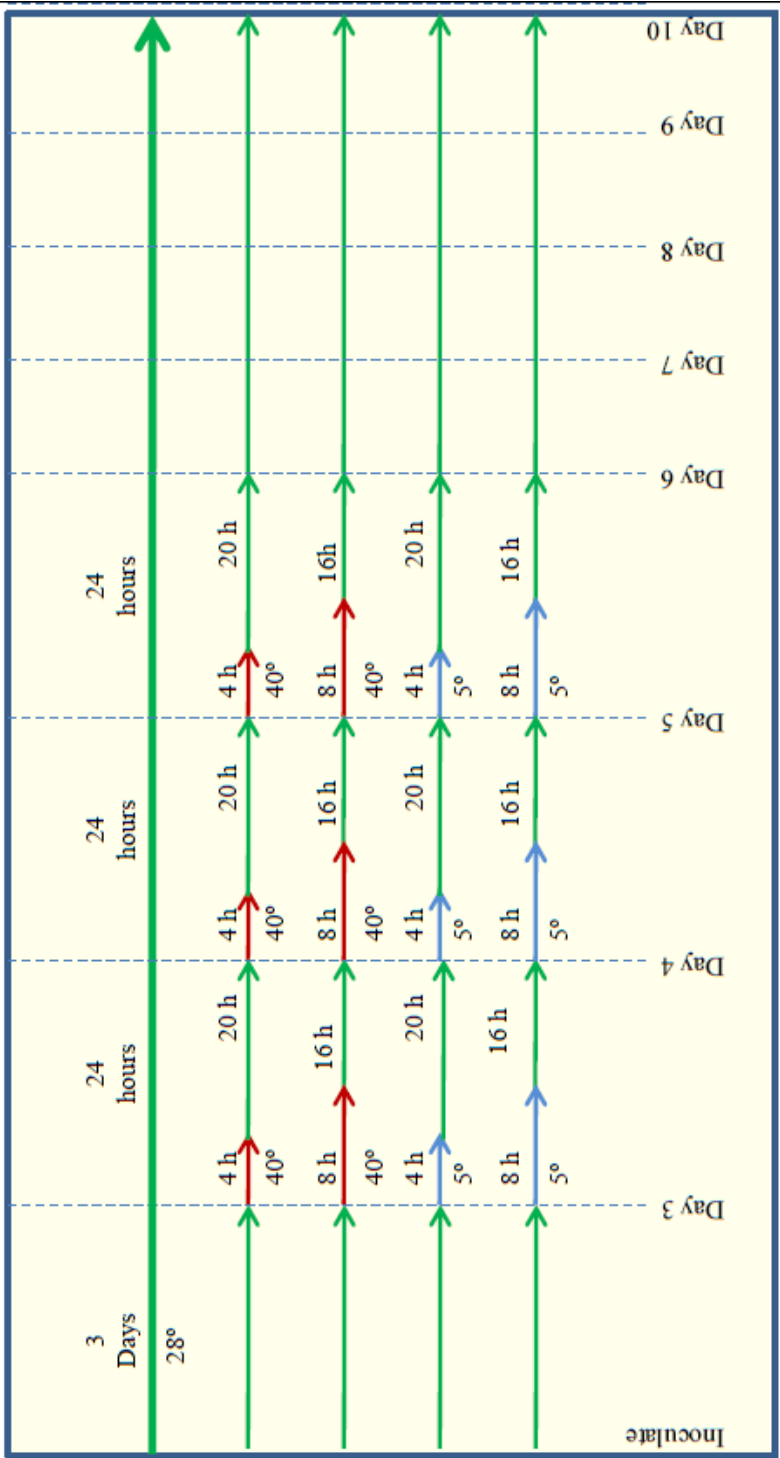


Figure 9.1 Visual representation of fungal isolate treatment plan: Five treatments with three replications were performed, for ten *Metarhizium* spp. isolates. Green arrows represent time exposed to 28°C; red arrows represent time at 40°C, and blue arrow represent exposure to 5°C. Perpendicular-dashed lines represent respective time intervals. Diameters of colonies were measured each morning beginning a 24 hour period; the Petri plates were immediately treated to their respective temperature regime as indicated.

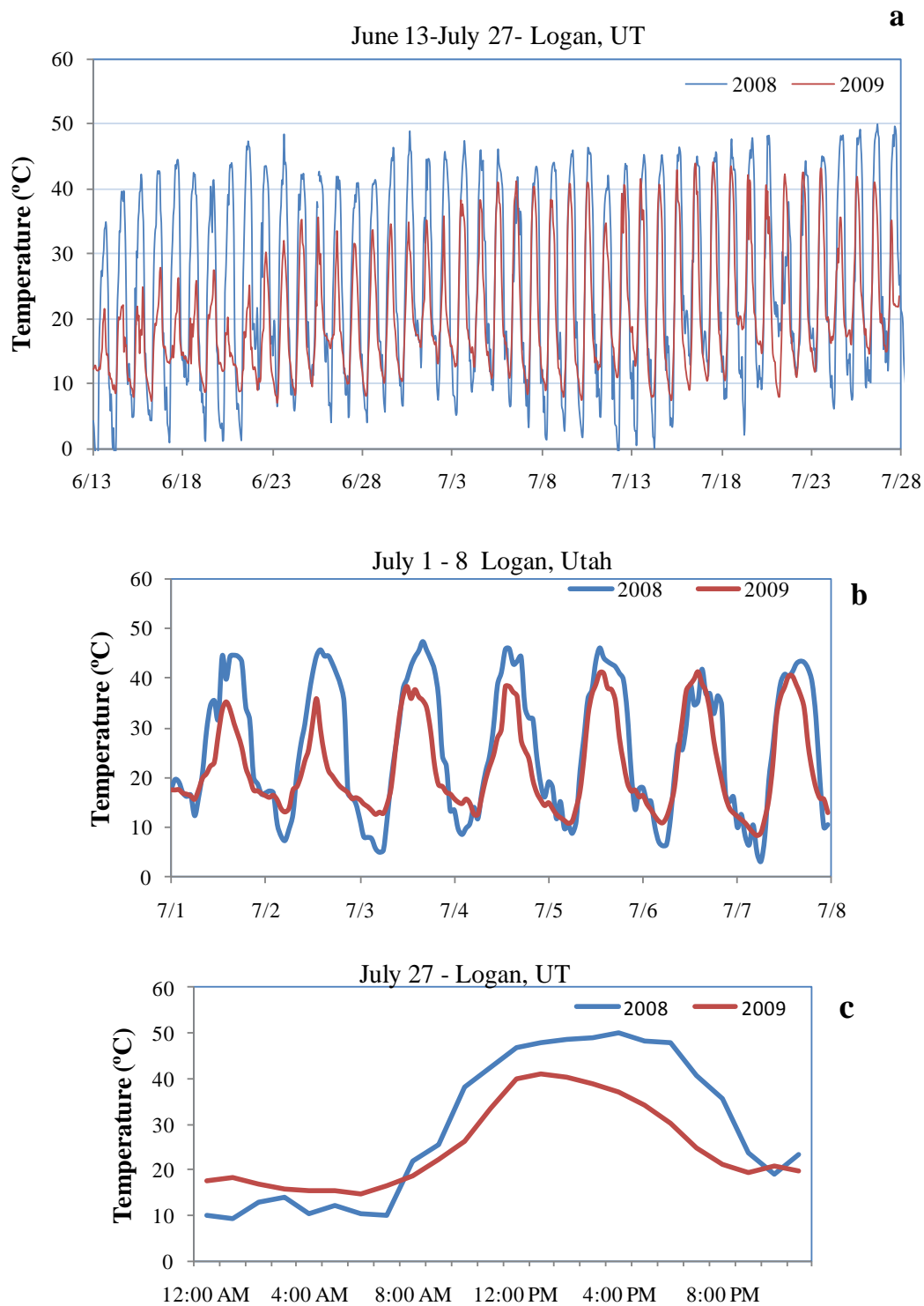


Figure 9.2 2008 and 2009 field-temperature data collected from the grasshopper surrogates in Logan, Utah, from (a) June 13 to July 27, (b) July 1 to July 8, and (c) July 27.

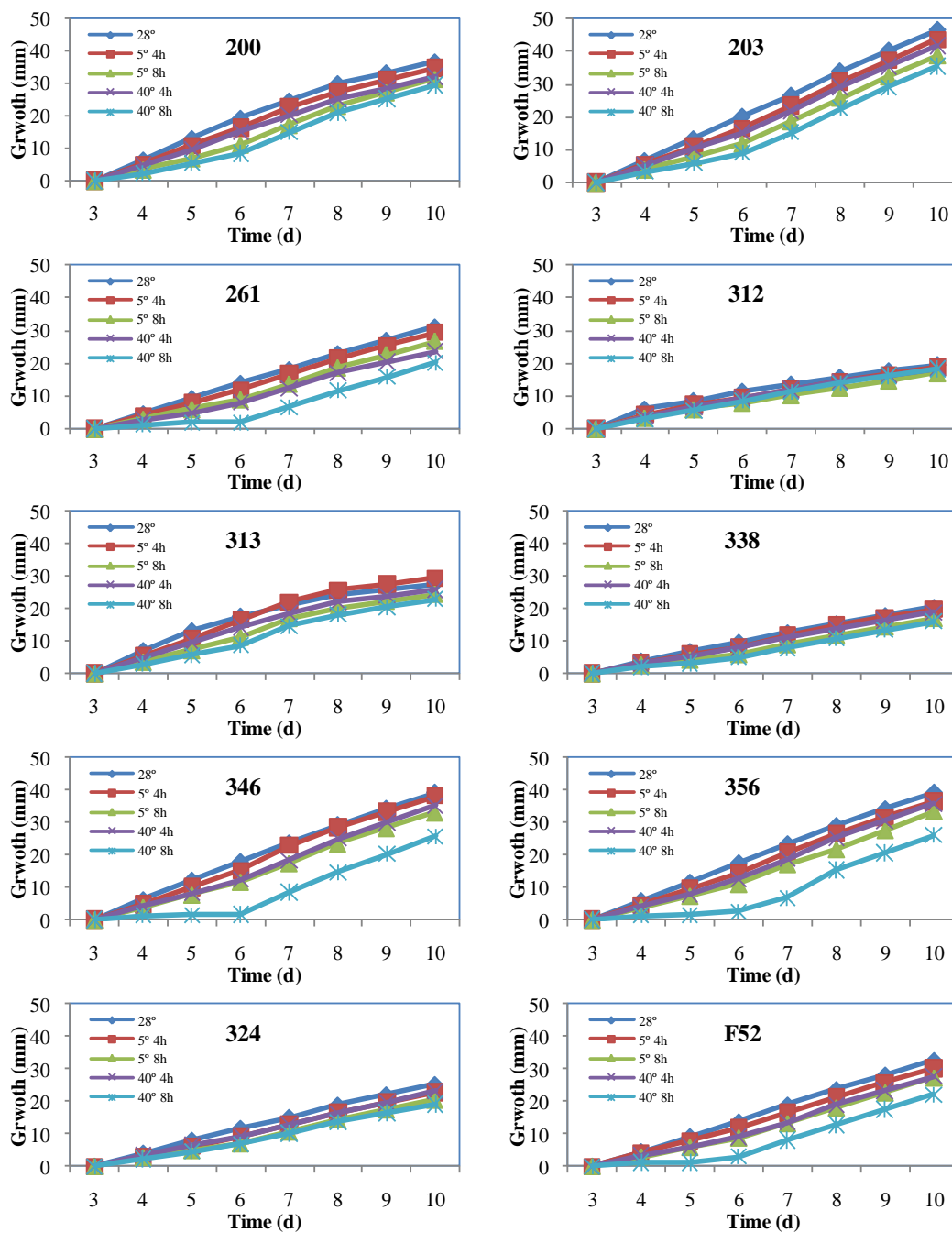


Figure 9.3 The average daily growth, as determined by measuring fungal-colony diameter daily of ten *Metarhizium* spp. isolates. The growth at day 3 (the beginning of temperature treatments) was taken as the zero point for constructing of the graphs. Five treatments replicated 3 times are represented for each isolate.

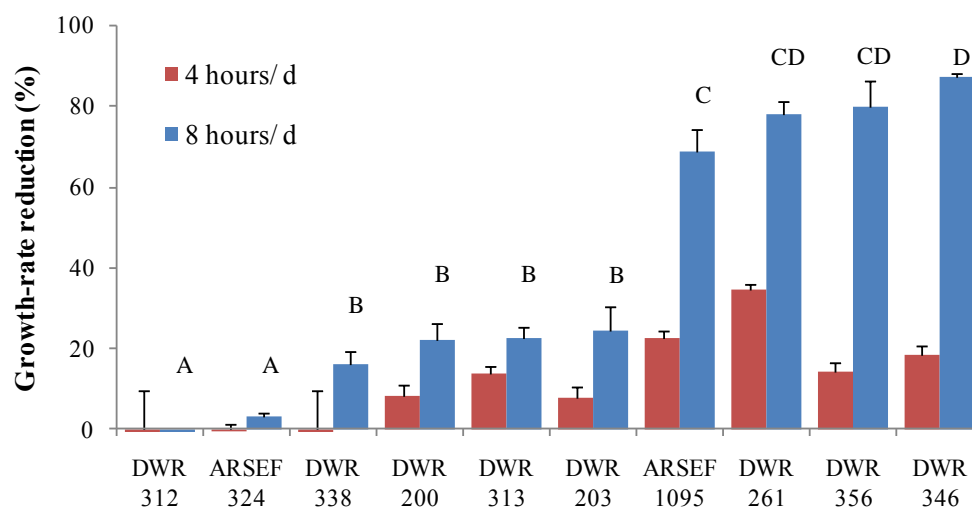


Figure 9.4 The % negative effect of the exposure to 40°C for either 4h or 8h of ten *Metarhizium* spp. isolates, as calculated by assuming that exposure to 5°C for an equal amount of time (4 or 8 hours) yielded the maximum possible growth rate (mm of growth per day) for that isolate. Percent reduction was calculated by dividing the growth rate of heat-treated colonies by the growth rates of cold treated colonies of the same isolate.

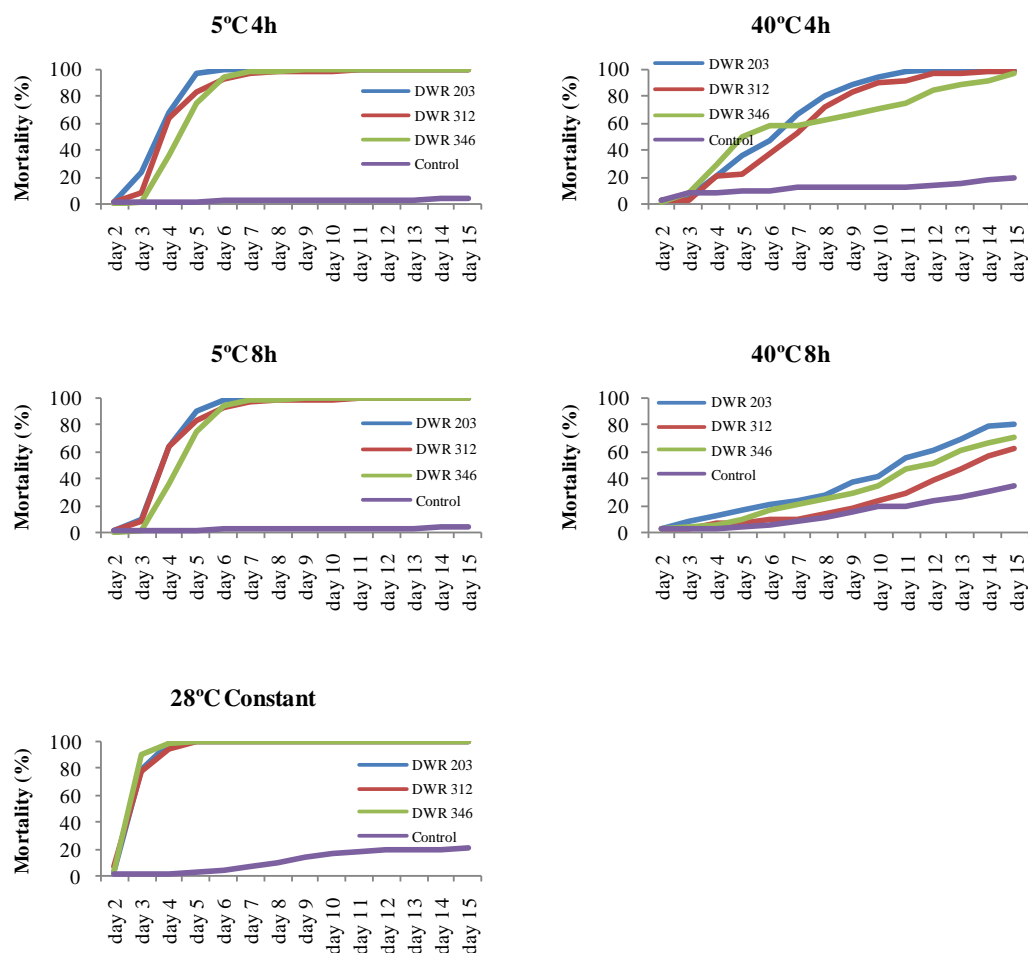


Figure 9.5 Graphic representations of daily mortalities of *Galleria melonella* larvae treated with conidia of three fungal isolates or with an aqueous tween solution (control). The larvae were held under one of five temperature regimes following the fungus exposure.

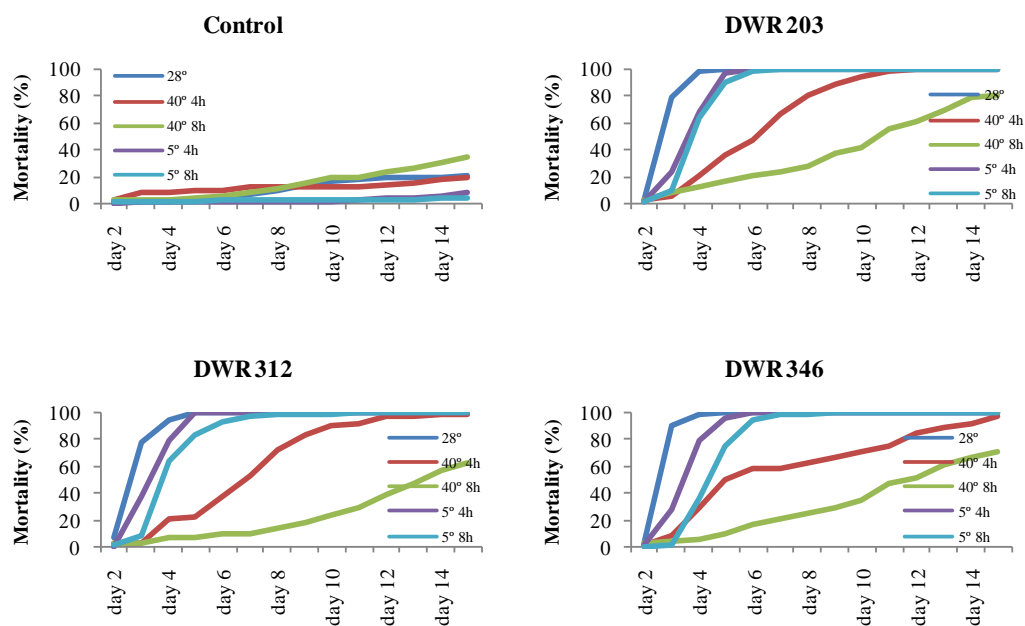


Figure 9.6 Graphic representations of daily mortalities of *Galleria melonella* larvae exposed to three *Metarhizium* isolates and then held at one of five daily temperature regimes.

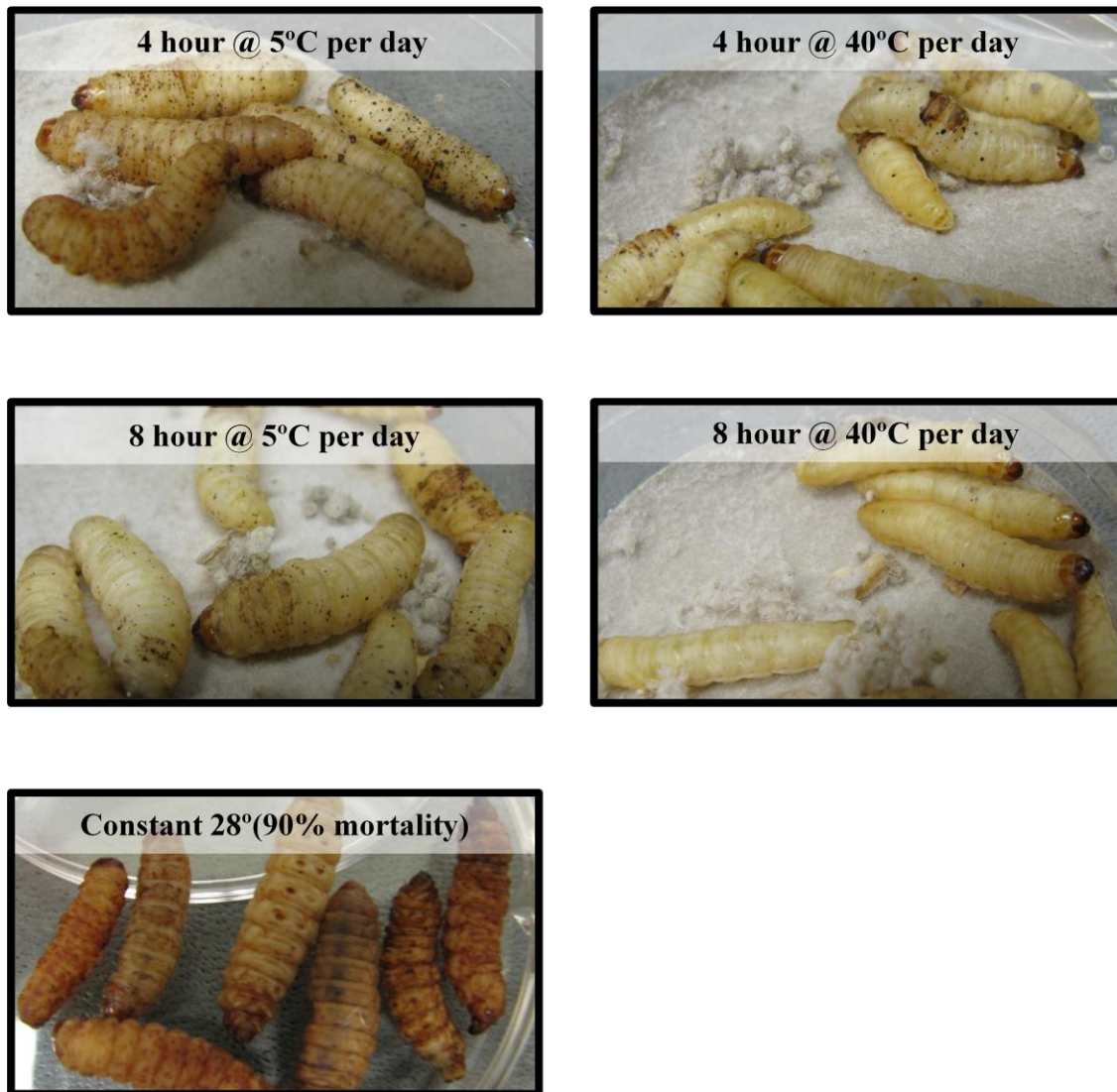


Figure 9.7 *Galleria melonella* larvae on day three after exposure to DWR 203 conidia at and then held at one of five daily temperature regimes. Note melanized fungus-infection sites.

CHAPTER 10

SUMMARY AND CONCLUSION:

DEVELOPMENT OF A LABORATORY BASED SYSTEM FOR SELECTING INSECT PATHOGENIC FUNGI WITH GREATEST POTENTIAL FOR SUCCESS IN THE FIELD

“No one supposes that all the individuals of the same species are cast in the same actual mould” (Darwin, 1859). Just as Darwin observed among plants and animals, different isolates of the same species of fungi have differing characteristics that individualize them as to their abilities to successfully infect hosts and reproduce. Mitosporic fungi (fungi that normally do not exhibit a sexual stage) acquire variability through mutations; and, as in the case of *Metarhizium*, possibly through parasexuality. If the variation is advantageous and heritable, it will be passed directly to offspring through asexual reproduction. The variability among individuals legitimizes screening to identify isolates possessing optimal characteristics.

Currently, developing an entomopathogenic fungus into a viable (truly useful) pest-control product in the USA is an onerous process. Tests in several areas will be needed. Issues include the challenge of *Safety*: to determine the effects of the fungal candidates on non-target organisms including mammals must be examined. These tests must be performed for each isolate individually. Another challenge is *Mass production*: The methods for mass producing the product often are specific to one or a few fungal isolates. The expensive and laborious input required to put a product on the market mandates that careful fungus-strain selection precedes commercialization. In part, Milner

(1992) attributes the success of *Bacillus thuringiensis*, the world's most successful microbial pesticide, to continued, near obsessive, screening for effective isolates.

A third major issue is *Ecological suitability*: Insects have adapted to very diverse habitats, with a wide range of climates and environmental conditions. Therefore, isolate selection should examine each strain's compatibility with the normal environmental conditions of a target pest(s). Isolates that pass this test presumably will possess traits that aid them in pest control in natural and/or agricultural settings. Furthermore, by identifying weaknesses that an isolate harbors, protocols can be devised to avoid or minimize those disadvantages (e.g., apply conidia at sundown to reduce first day UV and heat inhibition of infection inception). This will insure that disadvantages do not seriously affect negatively the control effort.

As of yet, of the entomopathogenic fungi isolated and evaluated, no single "silver bullet" isolate having all of the desired superior traits has been identified. Nevertheless, searches for superior fungal isolates traditionally have emphasized one or two traits (e.g., virulence) and it is very likely that this method has overlooked some potentially very valuable insect control fungi.

Overall Thesis:

The research reported here tests the **THESIS: A laboratory-based system can be devised that identifies, from among many *Metarhizium* isolates, those isolates with the greatest potential for successful biological control of pest-insects in the field.**

Addressing this thesis was accomplished by testing four hypotheses:

Hypothesis 1: Laboratory bioassays of target pest insects will distinguish highly virulent strains of *Metarhizium* from less virulent strains.

Milner (1992) suggested that selection of insect pathogens should be based primarily on bioassays, and he proposed a winnowing process to select the best isolates. This process involved a series of bioassays, the first being a very crude and comparatively quick assay using a single high dose of conidia that is aimed to identify from a large group those isolates that are pathogenic to a target insect. These isolates were then included in progressively more informative laboratory assays including, estimating LC₅₀s, simulated environmental-condition trials, and small scale outdoor field trials. Milner's paradigm focused on performance in bioassays, and accordingly he proposed that isolates with the best bioassay performance be selected for commercial use. Unfortunately, performing a series of bioassays against target insects is often impractical, for many reasons. For example, large laboratory colonies do not exist for many pest insects, making assays dependent on field-collected insects. Such insects may have unknown health issues or sub-lethal pesticide exposure prior to the experiment. Also, bioassays can be time consuming and labor intensive, thereby exhausting excessive amounts of laboratory funds for relatively little data. Also in some cases, virulence tests are not well correlated with field effectiveness (Roberts and St. Leger, 2004). Therefore they possibly can eliminate isolates that are well suited for field use, but are not well adapted to laboratory assays.

The bioassays performed in this study evaluated the adjusted percent mortality of Mormon crickets that occurred five days after treatment with an aqueous conidial

suspension (in Tween) of 1×10^7 conidia/ml (Chapter 3). Six of the ten isolates performed in an acceptable manner, causing high mortality by day five. ARSEF 324, the only *M. acridum* isolate included in this study, was not among those six. ARSEF 324 has previously been observed to be a virulent isolate when compared to other *M. acridum* isolates, and has already been commercialized and implemented in the field with success (Milner, 1997). It eventually kills all of the insects exposed to it, but due to very low toxin production, kills relatively slowly.

Testing an isolate for pathogenicity to a target pest should be a primary step when searching for a suitable new control agent. Based on the results of research presented in this thesis, primarily due to the complicated nature of implementing truly informative bioassays, and in accordance with Milner, I suggest that the preliminary selection step should be a rudimentary high-dosage assay to confirm that an isolate is indeed pathogenic to a target pest. Non-pathogenic isolates can be disregarded based on this test; but a low level of virulence, especially when virulence is measured in time (rather than dose) to kill, should not be an eliminating factor in selecting an isolate.

Hypothesis 2: Quantity and quality of mass-produced pathogenic fungi will vary among species and strains of *Metarhizium*.

“The inability to produce many biocontrol fungi en masse has ...hindered their development as biocontrol agents” (Auld, 1992). One of the chief drawbacks of developing fungal biological control agents for insect control is the delicate nature of large-scale mass-production, and difficulties in long-term storage of fungal product (usually conidia). Chemicals are often favored for pest-insect control because they are

inexpensive to mass produce and have a long shelf-life. The ability to produce large amounts of viable conidia on a low-cost substrate is a key factor in the success of a commercialized fungal product (Goettel and Roberts, 1992). Fortunately, many isolates of the entomopathogenic fungi *Metarhizium* and *Beauveria* are able to develop independently of their host, which makes large-scale production both technically and economically feasible (Feng et al., 1994; Grimm, 2001).

Using the methodology employed in this thesis research, the quantity and quality of mass-produced spores varied greatly among the fungal isolates examined (Chapter 4). Based on production quantities and viabilities, the best isolates for field use of those tested in this thesis are: DWR 338, ARSEF 324, DWR 346 and DWR 356. The range of production capabilities was vast, ranging from 0.64 grams/ 100g barley flakes (DWR 203) to 8.56g (ARSEF 324). This indicates that production capabilities cannot be ignored in selecting isolates for commercialization. Interestingly, the worst conidia producer (DWR 203) was among the most virulent in the bioassay evaluation, demonstrating the importance of performing multiple characterization tests. Many different factors are involved in the production of conidia; by altering the methodology, a poor spore-producing isolate may be encouraged to become more productive. This was seen with isolate DWR 203. In a separate test (data not shown), DWR 203 was grown on barley with increased air and decreased moisture; and under these conditions this isolate produced ~150% more conidia per 100g barley. This change was sufficient to include this isolate in a field trial performed in 2009 (Appendix). While production capabilities cannot be ignored and can potentially be a valuable tool for selecting between isolates

with other similar characteristics, selection should first emphasize performance in infecting the target insect and in the ability to survive harsh climate conditions.

Hypothesis 3: The tolerance to ultraviolet radiation will vary among species and strains of *Metarhizium*.

Sunlight can have variable effects on fungi. It can be stimulating, of no effect, or detrimental, depending on the fungal isolate and state of its development (Roberts and Campbell, 1977). For isolates of *Metarhizium*, sunlight, particularly UV-B, generally has a negative effect. The level of damage varies between isolates and species of *Metarhizium* (Braga et al., 2001a, 2001b; Rangel et al., 2004, 2006a; Rangel, 2006). Selecting an isolate with high tolerance to UV-B radiation will increase the likelihood that, in situations with high insolation, treated pest insects and/or the vegetation upon which they are feeding will result in development of infections in the target insect.

After 2 hours exposure to UV-B radiation (at an intensity comparable to noon sunlight in Logan, UT, in September), of the ten isolates tested in this thesis, the least susceptible was ARSEF 324 with 89.2% germination; DWR 346 with 77.9% germination, also demonstrated relatively high tolerance. Isolates DWR 313, ARSEF 1095, DWR 338 and DWR 200 were the most susceptible, realizing 28.3, 27.8, 27.1, and 10.6% relative germination, respectively. High light intensities are a relevant concern in the habitats of many pest insects (e.g., *Anabrus simplex*). Selecting an isolate that can tolerate and persist in the environment of the target pest is important. Clearly, evaluating tolerance to UV-B is a useful tool in gauging pest-control potentials of different isolates, and further helps researchers and applicators to make informed decisions while

developing biocontrol programs. Accordingly, UV-B-tolerance evaluations should be a primary consideration when selecting an isolate for field work.

Hypothesis 4: The effect of temperature on growth rates and survival of both *Metarhizium* spores and hyphae will vary among isolates and species.

Metarhizium species are generally considered to be mesophilic fungi, in that they have severely restricted growth below 10°C and above 37°C. The temperature can fluctuate between -4° to almost 50°C in temperate regions where many orthopteran pests (e.g., grasshoppers and locust) spread havoc. Selecting an isolate with rapid growth rates at permissive temperatures and with high tolerance to a wide range of both high and low temperatures will be fundamental to selecting a successful product.

This thesis includes four studies involving the effect of temperature on ten *Metarhizium* isolates. Two evaluated the growth capabilities (germination and vegetative) at several temperatures, one evaluated survival after exposure to extreme high temperature (45°C), and the last evaluated vegetative growth recovery after exposure to temperature stress. In each of these tests, variation among the isolates was significant, indicating that laboratory temperature evaluations are an effective way to distinguish isolates.

The studies that evaluated growth at various temperatures examined two traits: conidial germination, and vegetative (mycelia) growth. The germination study revealed that, of the *Metarhizium* isolates used in this thesis, DWR 203, DWR 261, DWR 346, DWR 356 and ARSEF 1095 germinated quickest; and, with the exception of ARSEF 1095, consistently were the fastest at germinating with the various temperatures (Chapter

6). The vegetative growth study revealed that between 20 to 32°C DWR 203 had the greatest rate of growth, followed closely by DWR 346 and DWR 356 (Chapter 8). ARSEF 1095 and DWR 261, the two isolates that performed well in the germination study but poorly in the vegetative growth study, are both *M. brunneum*. The sample size (two isolates) is small, but perhaps the attribute of germinating quickly followed by slow colony expansion is a characteristic inherent with this species. While these studies looked at different metabolic activities (viz. germination and mycelia growth), the characteristic examined in both is growth. The results for *M. brunneum* indicate that in some cases they are not redundant.

After four hours exposure to 45°C, ARSEF 324, DWR 356 and DWR 203 were the only *Metarhizium* isolates to have greater than 50% germination, followed closely by DWR 346. After exposure for six hours, only ARSEF 324 (*M. acridum*) had significant germination. *M. acridum* isolates are known tolerate higher temperatures than other *Metarhizium* spp. (Fernandes et al., 2010). Clearly, if the ability to tolerate high temperature for an extended period of time is a major concern in the normal habitat of a target pest then an *M. acridum* isolate is the best choice. This choice is possible, however, only if the target pest is an orthopteran insect, since the host range of *M. acridum* is basically restricted to this insect group.

The last temperature study evaluated the isolates' abilities to recover from heat stress. This experiment attempted to simulate fluctuating temperatures that occur in the field. Both high and low temperatures are known to stop fungal growth; however, the time required to reinstate growth when returned to normal temperatures is not known, and

possibly differs among isolates. Several studies have noted a delay in growth following some stress events (i.e., heat or UV radiation) (Zimmermann, 1982; Alves et al., 1996, 1998; Braga et al., 2001a, 2001b, 2001c). As predicted, all of the isolates when subjected to a cold temperature regime had their rates of growth decreased proportionally to the time spent away from the optimal temperature. At high temperature all of the isolates also exhibited reduced growth rates, with the rate reduction in some cases similar to that obtained with exposure for eight hours at 5°C /day (DWR 312 and ARSEF 324) while others (ARSEF 1095, DWR 261 DWR 356 and DWR 346) were considerably more sensitive to heat than cold (Chapter 9). Unexpectedly, the two isolates whose growth was most reduced by heat exposure were DWR 346 and DWR 356, both of which have high rates of growth compared to other isolates at 28°C. Isolates DWR 312 and ARSEF 324 were not affected by either regime of heat treatment. Interestingly, of the isolates tested, these two isolates had the lowest growth rates at 28°C. DWR 203 was affected by the daily transient heat exposure and lost, in comparison to the cold or control treatments, nearly 1 mm growth per day with 8 hours of daily heat exposure and 0.5mm/day with 4 hours daily exposure. Nevertheless, even with a reduced growth rate due to the heat regime, DWR 203 had a greater daily growth rate than either DWR 312 or ARSEF 324, isolates that did not exhibit a heat induced growth rate reduction. While none of the tested isolates were killed by exposure to the high temperature (40°C), the post-stress growth delay observed in this study is potentially devastating to field programs. Fungal disease development in pests that occur in regions with high temperatures is expected to occur at night when temperatures decrease. Unfortunately, isolates that require a prolonged

period to recommence growth after exposure to high temperatures are unlikely to have the opportunity to infect or to develop full-blown disease in its host.

Correlations

Basic Pearson-correlation coefficients were generated using the CORR procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows; *P*-values less than 0.1 were considered significant. The traits analyzed included: genetic grouping based on AFLP data; conidial size; the time required to reach 50% germination at 16, 28, 32, and 36°C; the rate of growth at 20, 24, 28, 32, 36, and 40°C; tolerance to 45°C wet heat for 2, 4, and 6 hours; tolerance to UV-B irradiation for 1 and 2 hours; conidia produced on one gram substrate (natural log transformed); the mortality observed on day five of bioassays; and degree (or absence) of the post-stress growth delay following daily four or eight hours exposure to heat (40°C). Randomly generating 441 correlation statistics likely will yield by chance correlated traits. Subsequently, traits that were not expected to be correlated will be identified as possible chance correlations.

A strong correlation was noted between the time required to reach 50% germination and the conidial size (Figure 10.1a); the Pearson correlation coefficient (*r*) was 0.781 (*p*=0.007) indicating that smaller spores tended to germinate faster. This was not expected and therefore may possibly be not be a true correlation.

There was also a strong correlation (*r*=0.682 *p*=0.03) between the post-stress growth delay after daily eight-hour heat exposure and the average growth rate at 24°C (Figure 10.1b); this correlation suggested that isolates with faster growth rates tend to take longer to recover after exposure to a heat stress. Possibly isolates that put more

resources into vegetative growth sustain greater damage during stressful periods, thus requiring more time to recover. Again this correlation was not expected.

A third correlation observed was between an isolate's tolerance to 45°C heat for four hours and an isolate's vegetative growth rate at 32°C ($r=0.681$ $p=0.03$); in this case, isolates that grew slower were less able to tolerate constant slightly elevated temperature for extended periods of time (Figure 10.1c). This correlation was not expected.

Another significant correlation was that isolates with high tolerance to 45°C also had high tolerance to UV-B irradiation ($r=0.715$ $p=0.02$) (Figure 10.1d). This finding may be used in reducing labor when selecting potential good field isolates by allowing the researcher to generalize an isolate's tolerance to environmental stress by testing only one of the two stressors. This correlation was expected, many of the attribute that enable an isolate to tolerate one stress also apply to the other stress.

Finally, a slight correlation was observed between the time it took to reach 50% germination and the percent mortality observed on day five in bioassays ($r=-0.589$ $p=0.095$). This suggests that isolates that germinated faster also had increased ability to infect and cause mortality (Fig 10.1e). several other authors have noted this correlations between growth or germination rates and virulence (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980; Hassan et al., 1989; Samuels et al., 1989).

Interestingly, no apparent correlation was observed between the speed of germination and the rate of growth when all ten isolates were included in the analysis; but if the *M. brunneum* isolates (DWR 261 and ARSEF 1095), were removed a strong correlation was evident ($r=-0.861$ $p=0.006$); this correlation indicates, as expected, that

fast-germinating isolates can be expected to have also means fast growth rates. The *M. brunneum* isolates were among the fastest germinators, but they had the slowest growth rates. In general, correlations based on genetic grouping (and on most other traits) were weak, or at most not strongly observed. Possibly this is due to the few isolates representing each genetic grouping, if more isolates from each species were studied possibly greater specie traits would be identified.

Conclusion

The objective of this thesis was to develop a series of laboratory experiments that could be used to identify isolates of entomopathogenic fungi with high potential for success in the field, and thereby avoid the excessive cost of individually testing each new isolate in field trials. Field trials by nature are complicated and difficult to conduct; they often involve many layers of variability which can render unequivocal interpretation of test results impossible. Also, uncontrollable weather conditions can cause delays or skew data in a direction not truly representative of typical conditions; and overcoming this would require several trials at different times, perhaps over many years. In addition, care must be taken to preserve realistic conditions in the trial set up, while simultaneously performing it in a quantifiable manner; these actions often increase cost and labor requirements beyond a practical level.

Laboratory tests to estimate the potential effectiveness of an isolate in the field generally have focused on virulence assays. In fact, Milner (1992) stated that a “bioassay against the target insect is... the only way to determine the activity of... new isolates”. Nevertheless, based on the results of this thesis and those of some other

studies, laboratory virulence tests are not necessarily the most useful indicator of potential field activity (Roberts and St. Leger, 2004). Potentially harmful environmental factors, such as UV-B radiation and high temperatures, also should be considered seriously when selecting new isolates.

The exhaustive methodology used in characterizing the ten isolates of this thesis is not a reasonable option for testing hundreds of new fungal isolates. At the same time, simplifying too much or winnowing out “poor” isolates based on insufficient data can lead to discarding potentially good isolates. I suggest a combination of simple tests that will strongly suggest which isolates might be “good” field isolates without requiring an unreasonable expenditure of resources. For example:

- 1) In all isolate selection surveys, use a simplified pathogenicity test to identify which isolates are pathogenic towards the target pest (Chapter 3). Detailed virulence assays are not necessary at this stage of the inquiry. A pathogenicity test can be accomplished by simply preparing a fungal suspension (quantified) and inoculating several individuals of the target pest insects. Treated-insect mortality should be compared with that of control (untreated) insects. Of the ten isolates in this study, all proved pathogenic towards Mormon crickets.

- 2) In addition to the pathogenicity evaluation, two or more other characteristics should be tested. These tests should be based on the climate and geographic location of the intended biological control program, e.g., if a pest insect inhabits a region with fluctuating temperature, tests should be selected that evaluate an isolate’s response to temperature extremes relevant to that area. For example, if a pest insect exhibits

behavioral fever as a defense mechanism (as do many grasshopper species), tests that evaluate survival after high temperature exposure should be conducted. Also if a program already has a standard fungus mass-production protocol, include this protocol in culturability tests.

The following series of general tests are aimed at quickly identifying fungal isolates with high potential for field use, but they are not meant to provide comprehensive characterization of the isolates. As an example the following protocol is proposed for identifying a *Metarhizium* isolate that will reduce Mormon cricket populations in the field. This insect lives in a temperate region with both high and low daily temperatures that can inhibit fungal growth, and strong sunlight that can inhibit fungal growth. Environmental conditions, therefore, are likely to be a major concern in isolate selection.

First, a test to identify an isolate's tolerance to wet heat should be conducted (Chapter 8). This can be done by exposing conidial suspensions to 45°C for 4 hours; isolates with greater than 40% germination after this exposure are considered heat tolerant. Note that for *M. acridum* isolates, a more appropriate test would be to select an isolate with that had greater than 60% germination after 12 hours heat exposure (Fernandes et al., 2010). Of the ten isolates in this study, ARSEF 324, DWR 356, DWR 203, and DWR 346 achieved greater than 40% germination (Figure 10.2a).

Second, test tolerance to UV-B irradiation (Chapter 5) by exposing isolates for 2 hours to UV-B radiation and consider isolates that achieve greater than 40% germination as UV-b tolerant. From this study isolates, ARSEF 324, DWR 346, DWR 203, DWR 312, DWR 261 and DWR 356 were tolerant to the UV-B exposure (Figure 10.2b).

Third, test the speed of conidial germination by measuring percent germination of each isolate on PDAY plates after incubation at 32°C for 12 hours (Chapter 6). Isolates with germination greater than 50% should be classified as fast germinators. Additional temperatures can be included in this study if fluctuating temperatures are a concern in the proposed treatment area. Of the ten isolates in this thesis, DWR 203, ARSEF 1095, DWR 261, DWR346, and DWR 356 had greater than 50% germination after 12 hours (Figure 10.2c).

Fourth, determine the rate of growth at 32°C. Isolates with rates greater than 4 mm per day on PDAY should be considered fast growers (Chapter 7). Isolates considered fast in this study included, DWR 203, DWR 346, DWR 356 and ARSEF 324 (Figure 10.2d).

Fifth, examine the mass production potential of each isolate on an inexpensive substrate (Chapter 4); ideally this will be done using a nutrient source appropriate to the region where a commercial product would be produced, and can be done on a small scale for test purposes (i.e., 100g substrate). Isolates producing more than 5.0×10^8 spores per gram of substrate should be considered good spore producers. This includes DWR 338, ARSEF 324, DWR 346 and DWR 356 in this study (Figure 10.2e).

Sixth, a test of the post stress growth rate reduction occurring because of exposure to 40°C for 8 hours should be considered (Chapter 9). Isolates with a growth-rate reduction of less than 30% of the adjusted control rate should be considered good isolates. Of the ten isolates in this study, DWR 312, ARSEF 324, DWR 338, DWR 200, DWR 313, and DWR 203 met this qualification (Figure 10.2f).

By performing the above series of simple laboratory tests, at least a somewhat informed decision can be made as to which isolates are likely to be the most successful in the field. Utilizing threshold cutoffs, and rating isolates either positive or negative based on those thresholds, helps avoid the complication of calculating analyses of variances. The threshold cutoffs presented in this thesis were determined by examining the findings on the test of all the characteristic traits and conducting analysis of variances among the various traits to estimate the level of determination that adequately separated the “better” (or even “best”) performing isolates from the remainder. The temperatures selected for each study were based on the requirements needed for a Mormon cricket control agent, since this afforded increased separation between the isolates under observation. Further consideration is being given to minimizing the effort involved in the screening tests so that more isolates can be evaluated.

Based on the suggested program above, isolates were ranked based on the number of tests for which they scored positively. Four of the isolates, DWR 203, DWR 346, DWR 356 and ARSEF 324, achieved positive scores on six of the seven evaluations (Figure 10.3). This suggests that these four isolates would be the best on which to move forward in developing a new biological control agent. Interestingly, none of the isolates scored positively in all areas tested; also no isolate failed to score in at least two areas. This further advances the concept that multiple tests definitely are needed. The heat tolerance and vegetative growth-rate tests seemed to be the most accurate at predicting which isolates were the best. The vegetative growth-rate evaluation was less clear, since several of the isolates were just below the threshold. Future attempts to identify good

isolates may choose not to include all of the proposed selection criteria presented here. I suggest that in addition to the pathogenicity test, which should be included in all surveys, heat tolerance is the most useful test and also should be incorporated into all evaluations.

As the territorial battle between man and insects continues, the sophistication of our weapons for protecting agricultural lands must keep pace with the endless ability of insects to adapt to, evolve around and overwhelm with sheer numbers our insufficient efforts. Synthetic chemical pesticides are widely used and have worked well in the past, but as our understanding of the complexity and fragility of ecosystems has increased, so has our moral and logical obligation to minimize human impact on the environment. In many circumstances, chemical insecticides are not the appropriate answer. Biological control is a viable alternative to pesticides, but if the paradigm of how we treat pest problems is going to change, commercial biological control agents must reduce in very obvious fashion the pest problem in the field. For this reason, selecting an isolate that will be successful is essential. By following the procedures outlined in this thesis, good isolate selection can be accomplished in the laboratory, and a successful isolate can be identified from the abundance of isolates present in nature.

Table 10.1 Based on the recommended protocol and thresholds for seven experiments, each of the ten *Metarhizium* isolates were scored on whether they met the threshold requirements (“x” indicates that the isolate did meet the threshold). Four of the ten isolates met the threshold requirements in six of the seven experiments.

Isolates	1. Pathogenicity	a. Heat Tolerance	b. UV tolerance	c. Germination	d. Vegetative growth	e. Culturability	f. PSGD	
DWR 200	x						x	2
DWR 203	x	x	x	x	x		x	6
DWR 261	x		x	x				3
DWR 312	x		x				x	3
DWR 313	x						x	2
DWR 338	x					x	x	3
DWR 346	x	x	x	x	x	x		6
DWR 356	x	x	x	x	x	x		6
ARSEF 1095	x			x				2
ARSEF 324	x	x	x		x	x	x	6

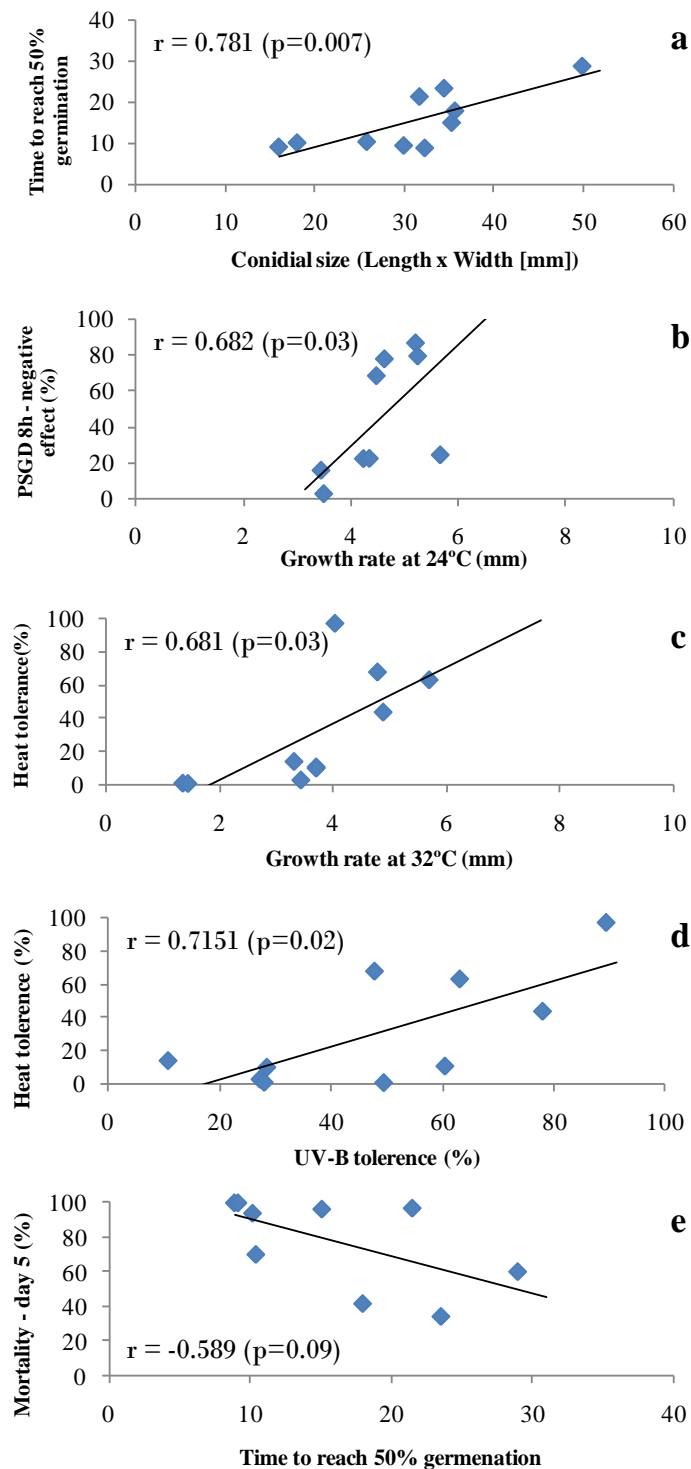


Figure 10.1 Pearson-correlation coefficients were generated using the CORR procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows; P -values less than 0.1 were considered significant.

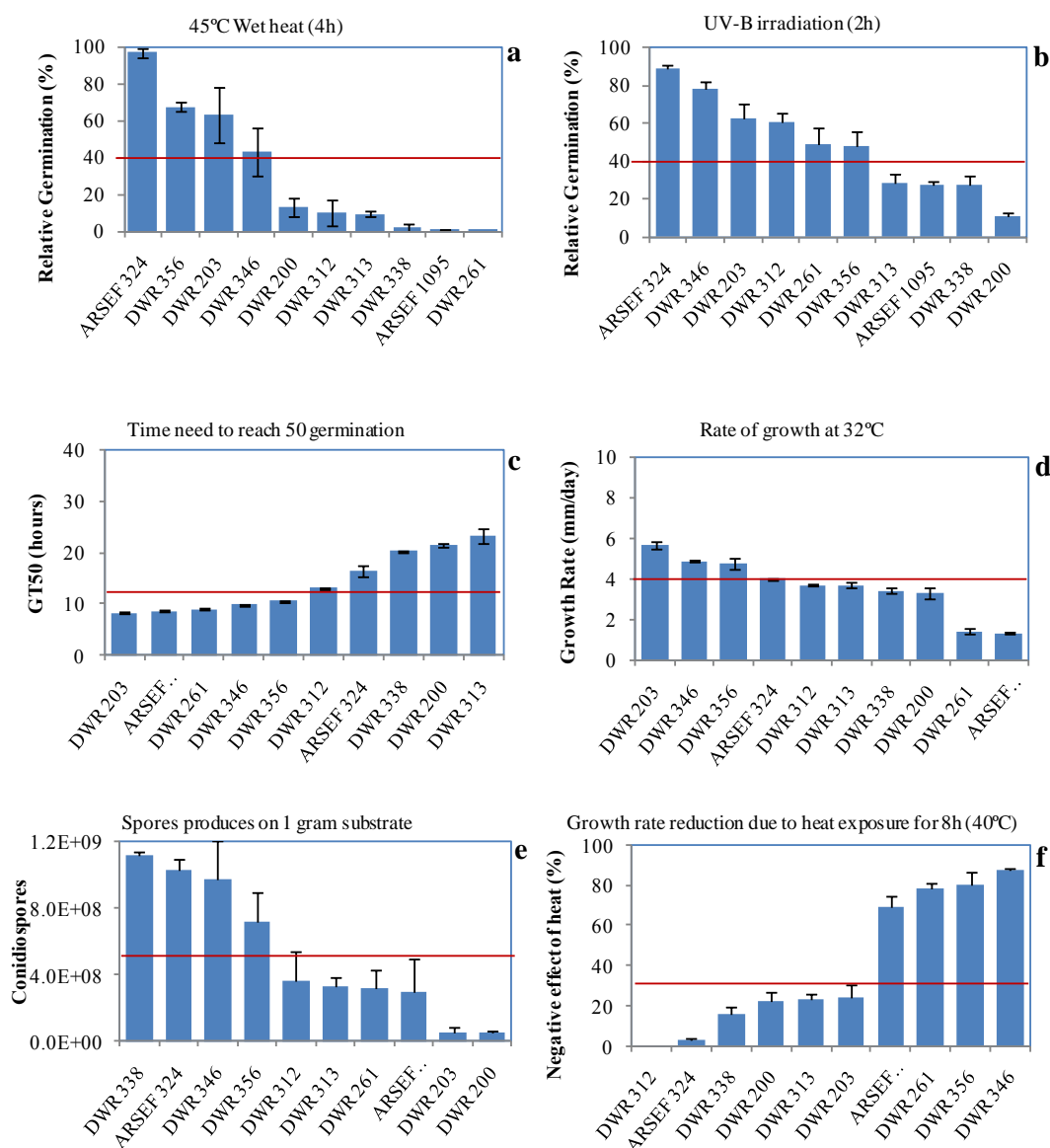


Figure 10.2 Graphical results of six experiments with ten isolates of *Metarhizium* illustrating a recommended protocol for screening new isolates. The red line indicates a threshold at which potentially “good” isolates can be distinguished from others.

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APPENDIX

Field-cage evaluation of *Metarhizium* isolates with high potential for field effectiveness towards the Mormon cricket (*Anabrus simplex*)

1. Introduction

Field evaluations of a biological control agent can provide realistic data that truly demonstrate a pathogen's potential effectiveness. Field trials attempt to evaluate success of a control agent interacting with a pest in the presence of many uncontrolled factors, including, unpredictable weather conditions, resource competition, natural predators, natural diseases, and biocontrol agent production/application methods. For a slow acting control agent, like *Metarhizium*, targeting a migratory insect, like the Mormon cricket (MC), the central problem is that treated insects have the opportunity to disperse before the control agent has a measurable field impact (Lomer et al., 2001). Many different strategies have been developed to overcome the problem of insect migrations while attempting to maintain true field conditions. These have included, in-field cage and arena trials, field application followed by cage incubation, green-island trial format, and large treatment areas. Because of the many factors involved in field trials, experiments normally need to be repeated over several years and at different locations before any level of confidence can be accredited to the results.

A further complication for testing biological control agents against the Mormon cricket is that the insects occur in large bands of individuals which mass together and move in the same directions (Gwynne, 2001; MacVean, 1990); so one area of land may be densely infested with MC, while a short distance away will be completely unpopulated. These constantly moving populations of crickets can hardly be sectioned

into treatment areas, and multiple bands are often difficult to locate. This increases experimental error due to varying population histories.

Because of the difficulty involved in carrying out experimentally sound field trials, it is important that resources be used to select promising biological control agents prior to field tests, rather than testing many isolates with unknown potential. The objective of this thesis was to present a system to identify through laboratory experiments, isolates that have high potential for field success. Of the isolates evaluated in this thesis, DWR 203, DWR 346, DWR 356 and ARSEF 324 were shown to be the most likely to achieve a high level of pest control in the field. Accordingly, the following study evaluated the efficacy of DWR 203, DWR 346, and DWR 356 for control of *A. simplex* in a field-cage trial, and compares these results with that of ARSEF 1095, a commercial isolate. ARSEF 324 was not field tested because it is an exotic isolate from Australia which is not currently permitted for outdoor use in the USA.

2. Materials and Methods

2.1 Fungal isolates:

Four *Metarhizium* isolates were used in this study; DWR 203 (AZ), DWR 346 (AZ) and DWR 356 (UT), and ARSEF 1095 (Figure 2.1). ARSEF 1095 was acquired from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA), and is a subculture of the same Austrian culture developed commercially by Novozymes Biologicals, Inc. The other three isolates were acquired from soil samples in Arizona and Utah. According to the methods described by Bischoff et al. (2009), ARSEF 1095 is classified as an *M. brunneum* isolate,

DWR 203 is an isolate of *M. guizhouense*, and DWR 346 and DWR 356 are isolates of *M. robertsii*. Stock cultures of each isolate were maintained at 4°C in test-tubes slants of potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco) (PDAY) adjusted to pH 6.9.

2.2 Conidial production:

Solid-substrate fermentation was accomplished using a three step method as described by Julie Grace at a solid-substrate-fermentation workshop presented at USDA/ARS/NPARL Sidney, Montana (2005).

Step 1: Fungal colonies were produced on 23ml of potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco)(PDAY) adjusted to pH 6.9 media in 95 x 15 mm polystyrene Petri dishes and incubated at 28°C.

Step 2: A blastospore culture was produced in liquid media. The medium consisted of glucose 10 g; yeast extract 10 g; K₂HPO₄ 4 g; KH₂PO₄ 2 g; NH₄NO₃ 1 g; MgSO₄ 0.2 g, KCl 0.2 g; FeCl₂ 0.002 g; MnSO₄ 0.002 g; ZnSO₄ 0.002g; and 1 ml of gentomycin (10 mg/ml) per liter medium. This medium was autoclaved in an Erlenmeyer flask with a cotton plug in the mouth to allow air exchange, cooled, and inoculated with conidia produced in step one. The flasks were placed on a gyratory shaker (172 rpm) and allowed to grow for 5 days at 24°C.

Step 3: In a polypropylene autoclave bag (8 x12cm Fisherbrand), 100 grams organic flaked barley (Shangri-La Health Food, Logan, Utah) were added along with 60 ml distilled H₂O. Bags were closed around a 10 cm cotton plug with a twisty-tie, and

autoclaved for 30 min at 121°C. Bags were allowed to cool, opened in a sterile chamber, and 30 ml of blastospore culture for each isolate was added. Inoculated bags were placed at 28°C \pm 1 and allowed to grow for 14 days with daily massaging to break up clumps. Because of low conidial yield using this method for DWR 203 and ARSEF 1095, the methodology was altered. Moistened barley was placed in metal pans, covered with aluminum foil and autoclaved for 30min. The pans were then inoculated with the blastospore culture prepared in step two and incubated at 28 \pm 1°C. Each day, pans were removed to a laminar flow hood and stirred using a sterilize spatula. Conidial yields were greatly enhanced through this method.

After solid substrate fermentation was completed, each fermentation bag was emptied into double-paper brown paper lunch sacks (Giant size, lunch bags, Kroger CO., Cincinnati, Ohio, USA); the opening was folded 3 times, closed with staples and dried for 5 days at ~24°C. After drying, conidia were separated from substrate using a series of mesh sieves (#20 and #100, Fisher Scientific) placed in a sieve shaker for 15 min.

2.3 Inoculum preparation

Conidial production for all four isolates, DWR 203, DWR346, DWR 356 and ARSEF 1095, was accomplished in Logan, Utah. Due to persistent contamination challenges, DWR 356 was not successfully produced in 2009, so year-old spores produced in 2008 and dry stored at 2°C were used in the 2009 field trial. The viability of these spores was 72%. Viabilities of dry spores prior to conidial formulation in oil for DWR 203, DWR 346 and ARSEF 1095 conidia were 75, 96 and 94% respectively.

Formulation consisted of suspending in food grade canola oil, and concentrations adjusted to $\sim 2.5 \times 10^9$ viable spores per ml.

2.4 Field treatment:

Treatments were applied using the Field Aerial Application Spray Simulation Tower Technology (FAASSTT), a system developed by R. Nelson Foster (USDA-APHIS-PPQ-CPHST-CPHST laboratory Phoenix, Phoenix, Arizona.). This system simulates rates and droplet size of a typical aerial application in a contained 0.5 meter area. The system involves a mobile spray tower created from a cardboard cement form (5ft \times 2ft) with an airbrush (Paasche type H with 75 regulator) modified for liquid injections mounted in the tower's top.

A 0.5 acre plot of agricultural field was provided by Utah State University for the field-cage trial. The field, located at N 41°45'37.39, W 111°48'42.54, was planted with alfalfa and grass (Figure A2). Prior to beginning the trial, the vegetation was cut to 5 inches plant height. Six blocks were designated for treatments; within each block, rows were individually assigned to one of five treatments. Treatments consisted of either control (treated only with pure canola oil), or one of the four fungal treatments. Each row contained fourteen 0.508 meter diameter treatments plots marked with aluminum rings and separated from each other by approximately 1.5 meters. Treatments were applied using the FAASSTT system. Because of the time it take to spray a single block and repeated rainy weather conditions, not all blocks were treated at the same time. Block one was treated on June 9, blocks two, three, and four were treated June 12, block five was treated June 13, and block six was treated June 14, 2009 (Figure A1).

Following each fungal (or control) treatment, a 22 cm high \times 17.8 cm diameter cage was placed in the center of the treated area. Cages were constructed from 1/8 inch steel hardware cloth (ACE hardware). Immediately after cage placement, an individual field-collected 4th instar Mormon cricket (MC) was placed in each cage. On rainy days a paper plate was secured on top of each cage to prevent fungal inocula from being washed away. Field cage mortality was checked daily for 40 days.

To confirm that a lethal dose of pathogen had been applied, after 48 hours, MC from two randomly selected cages from each row were brought to the laboratory and incubated at 24°C. The MC mortality under the moderate light and temperature condition of the laboratory was checked daily for eight days.

2.5 Statistical analysis:

A survival analysis was used to evaluate the effect of fungal treatments on the Mormon crickets in the field. Because of high control mortality after day 30, insects surviving beyond day 30 were considered censored. Statistically significant differences between fungal treatments and control treatments were calculated based on the Cox Proportional Hazards Model; *P*-values less than 0.05 were considered as significant. Statistically significant variation between blocks was not seen when data from block one were excluded from the analysis due to high control mortality in on block. Data analyses were generated using the PHREG procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows.

3. Results:

Mormon crickets that were brought to the laboratory 48 hours after treatment died, confirming that the fungal dose was lethal and that canola oil without fungal conidia did not cause mortality. By day eight, all of the MC from fungal treatment cages were dead, while no control insects died within this time frame.

Mortality in all fungal treatments in the field varied significantly from the control treatments; fungal treatments induced insect mortality. By day 30, DWR 346 had killed nearly 60% of the treated MC, DWR 203 51% mortality, DWR 356 44% and ARSEF 1095 43% mortality (Figure A3). The control mortality on day 30 was 22%. The only statistically significant difference among the fungal treatments was between DWR 203 and ARSEF 1095 ($p = 0.04$).

4. Discussion:

Integrated Pest Management (IPM) stratagems focus on reducing ecological and economical losses resulting from pest insects. In most circumstances, pest insects do not need to be eradicated. Rather, if populations can be adequately reduced, economic losses can be avoided. Both DWR 346 and DWR 203 reduced the MC populations by greater than 50%. While this is perhaps not as efficient as a chemical insecticide, it is likely sufficient to avoid severe crop or rangeland damage.

Chemicals are effective at suppressing pest populations, but they have many disadvantages, including: they are prohibited in many areas, such as near waterways and habitats of threatened or endangered species; their use by organic farmers and ranchers is not allowed; they are under continual review by government and private agencies for any

unforeseen harmful side-effects to humans or other mammals; and they generally have a short effective period after field application. Furthermore, broad-spectrum insecticides often will affect non-target beneficial insects, including pollinators, resource producers (honey, silk or wax), natural enemies of insect pests, wildlife food, and scavengers (Lomer et al., 2001; Murphy et al., 1994; Peveling et al., 1999). Having an entomopathogenic fungus available for use in situations where chemicals are not appropriate would be highly beneficial.

In this field-cage trial, the vegetation, not the insect, received the fungal treatment. All of the Mormon crickets from the treated plots that were removed from the field after 48 hours and incubated in the laboratory died within eight days, demonstrating that a lethal dose was acquired from the vegetation. The slower mortality observed in the field was caused by the additional variables present in the field. Environmental conditions have major impacts on the efficacy of insect pathogens.

Weather was likely a factor in the results seen during this field trial. For the first several weeks of the trial, temperatures were cooler than normal and did not exceed 35°C. It is not clear if this would be a benefit or a disadvantage to the infection process. While the possibility of high-temperature stresses delaying the infection process was not a concern during this period, the low temperatures likely reduced germination and slowed fungal growth. On the other hand, the sky was overcast reducing conidial exposure to UV radiation, but there was a high level of moisture which encourages germination. There was considerable precipitation during the experiment, which could possibly wash away the fungal inoculums, and the covering plates used occasionally to protect from rain

altered host behavioral fever. This just further illustrates the complications involved in field work.

Based on the results of this field trial, DWR 346 is statically more virulent in the field than the commercial isolate ARSEF 1095. DWR 203 also had a faster rate of kill than ARSEF 1095. While DWR 356 had nearly an identical rate of kill as ARSEF 1095, the DWR 356 conidia used in this trial were over a year old, which likely reduced their virulence. Preliminary laboratory evaluations can effectively select isolates with good potential for the field. The three DWR isolates were predicted by the laboratory evaluation to be “good” field candidates while ARSEF 1095 was not. The results of this field trial confirm that the process used to identify these isolates as promising control agents was effective. By evaluating more isolates in the laboratory, perhaps an even more effective field isolate might be found. Field trials are complicated and the results are often unclear; but they are necessary to confirm that isolates identified as promising biological control agents through laboratory tests do, in fact, work in the field.

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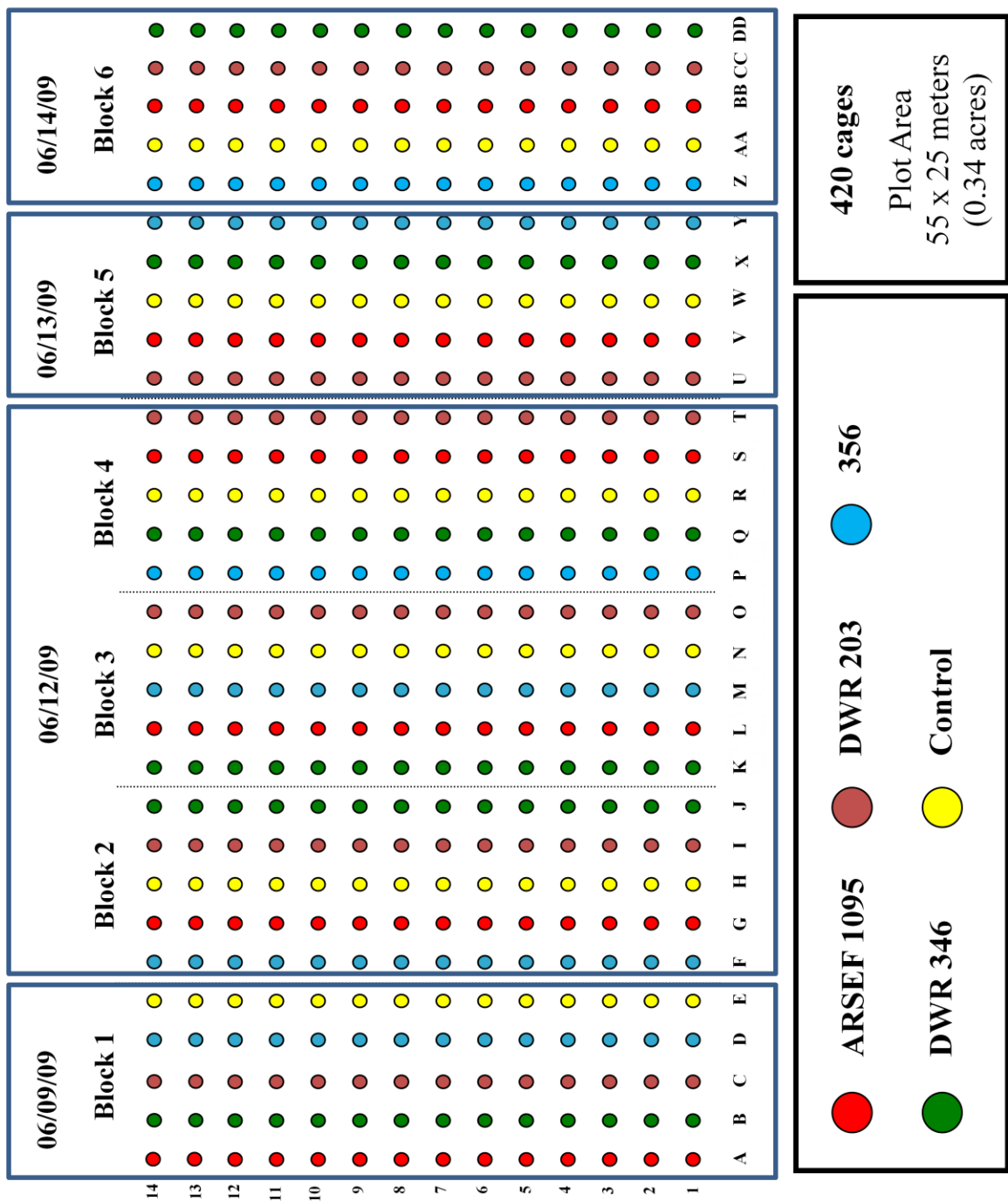


Figure A1 Diagram of the field layout: Fourteen cages per treatment in each of six blocks with treatments were randomized within the each block.



Figure A2 Alfalfa/grass mixed field in Logan Utah located at N 41°45'37.39, W111°48'42.54.

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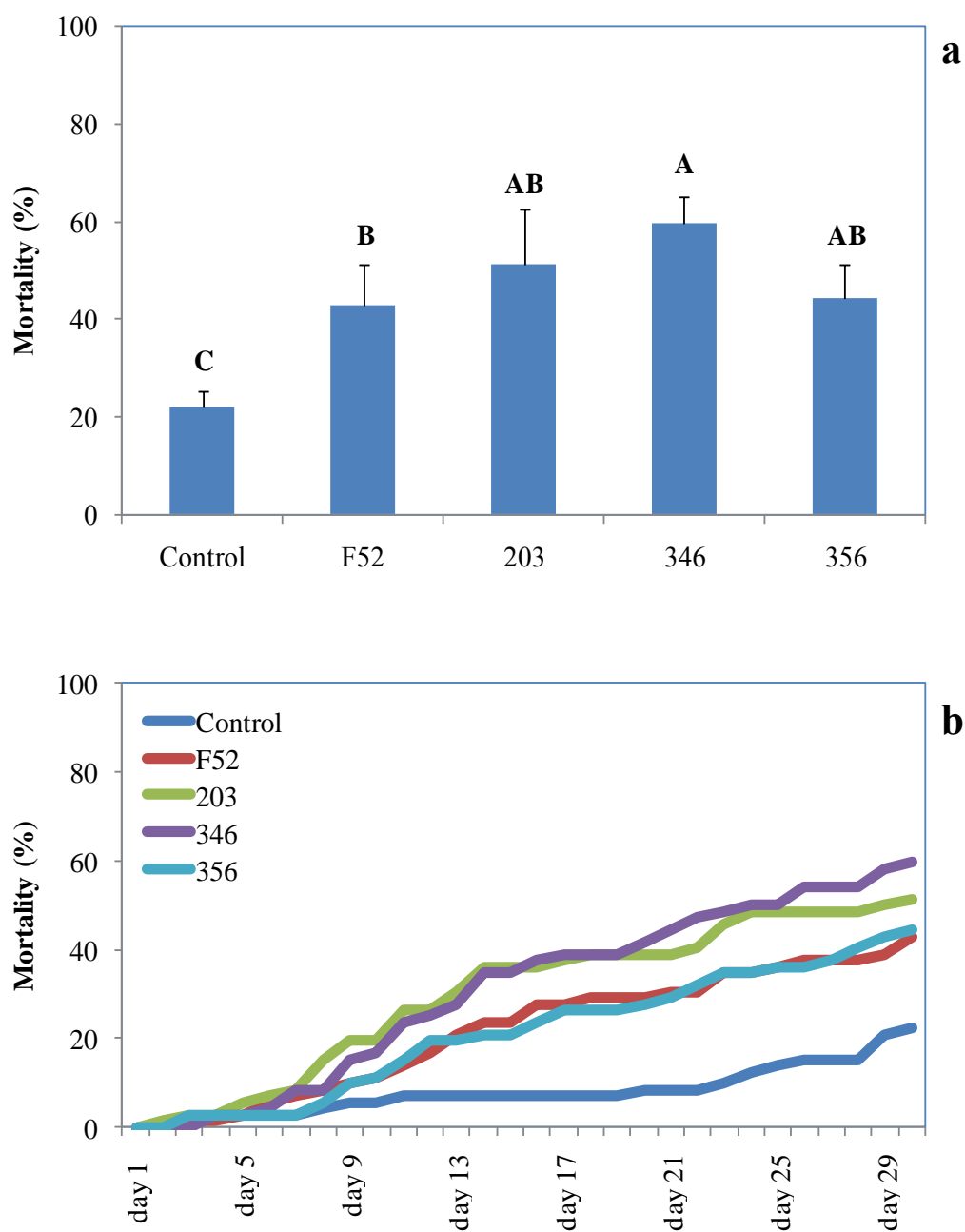


Figure A3 Mormon cricket mortality in the field. (a) The average mortality observed on day 30 for each treatment. Bars represent the standard error of 5 blocks. (b) Daily mortality from day one to day 30 for each treatment. Note: Due to high control mortality, block one results were not included in the statistical analyses.